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# Particle mediated co-delivery of IL-10 and antigen inhibits T cell activation but fails to induce tolerance

A thesis submitted by

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for the degree of

Doctor of Philosophy

2010

# Abstract

Immune disorders such as allergy and autoimmunity are becoming increasingly common in developed countries. Self-reactive T cells exist in both healthy and autoimmune individuals. It is generally understood that hyperimmune disorders are caused by insufficient regulation, namely loss of activity of regulatory T cells. Whilst regulatory T cells exist naturally it is also possible to induce them both *in vitro* and *in vivo*. Immunotherapeutic techniques aim to provide noninflammatory exposure of antigen to the immune system with the aim of inducing antigen-specific regulatory T cells.

Interleukin-10 (IL-10) is a cytokine with well known immunosuppressive qualities. It inhibits both the migration and the antigen-presenting ability of dendritic cells. It also has direct effects on T cells. Indeed, IL-10-secreting $T_R1$ regulatory T cells were identified almost 15 years ago; their *in vitro* generation being dependent on exposure to IL-10.

Particle-mediated DNA delivery (PMDD) is a promising method of immunisation and is especially suited to vaccines intended to have greater control over the response they induce. One of the main reasons for this is the possibility of including genes encoding immunomodulatory molecules alongside the antigen gene.

This study utilises a mouse model involving the adoptive transfer of TCR-transgenic $CD4^+$ T cells and establishes the response of these cells to PMDD immunisation. The model was then used to examine the effect of coadministration of the IL-10 gene. Its inclusion in the vaccine suppressed the response to antigen. This effect was maximal when the IL-10 gene was expressed in the same cell as the antigen gene. Using sequential immunisations the model was extended in order to study long-term effects, namely tolerance and the induction of regulatory T cells. Finally a mouse model of allergic asthma was used to examine any tolerogenic/therapeutic effects of the antigen-IL-10 vaccine. No significant long-term tolerance to antigen was identified.

These results demonstrate that whilst the presence of IL-10 clearly inhibits the T cell response to antigen it does not necessarily confer tolerogenic properties on these cells. This brings into question whether IL-10 in the periphery, supplied, for example, by $T_R1$ cells, generates fresh regulatory T cells or merely inhibits the response to a particular antigenic challenge.

# Acknowledgements

I am very grateful to Professor Benjamin Chain, my academic supervisor, and Dr Sara Brett, my industrial supervisor, for their support, guidance and advice during my studies. My exposure to both the academic and industrial research worlds will no doubt stand me in good stead for my future career. I would especially like to thank them for their patience during the many changes in my life that significantly delayed the submission of my PhD thesis.

I would also like to thank Dr Remi Creusot for his mentoring, especially with regard to the key animal model involved in this project, for his humour and for his friendship.

I had the pleasure of supervising Dr Daljit Sura, an intercalated medical student, during his research project. His interest and pertinent questions repeatedly caused me to stand back and think and were the source of several new ideas. I would like to acknowledge his contribution to some of the data in chapter 3.

At GlaxoSmithKline my thanks go to Dr Volker Germaschewski for his guidance on cloning and the development of plasmid vectors, Peter Topley for his teaching and guidance on the gene gun, Maria Feeney, Dr Olivier Vidalin and Dr Karen Barber for their training and help with animal work, Dr Jenny Craigen, Maria Daly, Dr Ian Catchpole and other members of my group for their help with cellular immunology and flow cytometry.

At the Windeyer Institute I would like to thank the staff and my colleagues for their friendship and help. They include Prof David Katz, Dr Jane Skok, Pamela Manfield, John Cheshire, Samantha Photiades, Ian Gerrard, Dr Gabriel Pollara, Dr Matthew Handley, Dr Rino Tulone, Dr Cheryl Chiang, Dr Ariel Rad, Dr Pippa Newton, Dr Paul Free, Dr Claire Swetman, Dr Helen Rowe and Dr Emma Willoughby.

This work would not have been done without the financial support of the Biotechnology and Biological Sciences Research Council and GlaxoSmithKline.

A special thank you goes to a friend and former employer, Mr Terence Francis, for providing me with the opportunity and resources to complete my thesis.

My parents, Gary and Laura, are the reason behind my enthusiasm for science and learning and I thank them for their love and support and for teaching me reason. My loving and devoted wife Cara has encouraged me and supported me no matter how difficult our lives have been. I cannot thank her enough for allowing me the opportunity to complete this work and for giving me two wonderful children, Yehoshua and Eden, in the meantime.

I thank God for the incredible phenomenon that is nature and for the puzzles within.

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# Abbreviations

| | |
|---|---|
| APC | antigen presenting cell |
| BSA | bovine serum albumin |
| CFSE | carboxyfluorescein succinimidyl ester |
| Complete [*medium*] | Supplemented with 10% heat-inactivated Foetal Calf Serum (PAA Laboratories GmbH, Haidmannweg, Austria), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (all provided by Cancer Research UK) and 60μM 2-mercaptoethanol (Invitrogen-Gibco 31350-010) |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | dendritic cell |
| ELISA | enzyme-linked immunosorbent assay |
| ELISpot | enzyme-linked immunospot (assay) |
| FACS | fluorescence-activated cell sorting |
| FCS | foetal calf serum |
| GSK | GlaxoSmithKline |
| i.v. | intravenous |
| IFN | interferon |
| IL | interleukin |
| LPS | lipopolysaccharide |
| MHC | major histocompatilibity complex |
| OVA | ovalbumin |
| PAMP | pathogen-associated molecular pattern |
| PBS | phosphate buffered saline |
| PBST | phosphate buffered saline containing 0.05% Tween 20 |
| PMDD | particle-mediated DNA delivery |
| TCR | T cell receptor |
| $T_H$ | helper T cell |
| TLR | Toll-like receptor |
| UCL | University College London |

# 1. Introduction

## 1.1. General introduction to interleukin-10

### 1.1.1. Discovery

IL-10, originally Cytokine Synthesis Inhibitory Factor (CSIF), was initially described as a $T_H2$ cytokine insomuch as it inhibited $T_H1$ cytokines[(Fiorentino *et al.*, 1989)] and $T_H1$ cells[(Macatonia *et al.*, 1993)] and promoted the humoral response[(Defrance *et al.*, 1992; Rousset *et al.*, 1992; Briere *et al.*, 1994)]. This $T_H2$-like quality continues to be confirmed[(Biswas *et al.*, 2007)] but IL-10 was soon understood to be a more general inhibitor of cytokine expression[(de Waal Malefyt *et al.*, 1991a; de Waal Malefyt *et al.*, 1991b; Fiorentino *et al.*, 1991a)]. Indeed, it has been shown to be produced by both $T_H1$ and $T_H2$ cells[(Del Prete *et al.*, 1993; O'Garra and Vieira, 2007)] and, under certain conditions, can actually be selectively inhibitory towards $T_H2$ responses[(Cottrez *et al.*, 2000; Moore *et al.*, 2004)].

Confirmation of the important immunoinhibitory role of IL-10 comes from studies of IL-$10^{-/-}$ mice. These mice naturally develop, or are more susceptible to induction of, a range of immune disorders such as microbe-induced colitis and other inflammations[(Kuhn *et al.*, 1993; Gazzinelli *et al.*, 1996; Sellon *et al.*, 1998)], autoimmune encephalitis[(Bettelli *et al.*, 1998)] and asthma[(Tournoy *et al.*, 2000)]. IL-$10^{-/-}$ mice suffer from exaggerated responses to inflammatory mediators[(Berg *et al.*, 1995a; Berg *et al.*, 1995b)]. In contrast, mice overexpressing IL-10 are protected from such responses[(Lang *et al.*, 2002b)] as are mice that receive the cytokine parenterally[(Howard *et al.*, 1993)].

Further proof of the importance of IL-10 in immunosuppression is the evolution of multiple viral homologues of the cytokine, collectively known as viral IL-10 (vIL-10). Epstein-Barr virus (EBV)[(Hsu *et al.*, 1990; Moore *et al.*, 1990)] and cytomegalovirus (CMV)[(Kotenko *et al.*, 2000)] are both viruses of significance to human health and encode very different vIL-10 molecules; the former being much more homologous to human IL-10. By inhibiting the immune response these vIL-10 confer an advantage on the virus[(Salek-Ardakani *et al.*, 2002; Chang *et al.*, 2004b; Raftery *et al.*, 2004; Jenkins *et al.*, 2008; Nachtwey and Spencer, 2008)]. The stability of the EBV vIL-10 gene across isolates further supports the argument that the importance of IL-10 has driven the evolution of viral homologues[(Kanai *et al.*, 2007)].

### 1.1.2. IL-10-producing cells

As described in 1.1.1, IL-10 is commonly known to be produced by $T_H2$ $CD4^+$ T helper cells. However, IL-10 can be produced by $T_H1$ cells[(Jankovic *et al.*, 2007; Trinchieri, 2007; Rutz *et al.*, 2008)] as well as both $CD4^+CD25^+$[(Maloy *et al.*, 2003)] and $T_R1$ regulatory T cells (reviewed in [(Roncarolo *et al.*, 2001)]). $CD8^+$ T cells[(Tanchot *et al.*, 1998)] and B cells[(O'Garra *et al.*, 1990; Fillatreau *et al.*, 2002; Mauri *et al.*, 2003)] are also known to express IL-10. As well as lymphocytes, myeloid cells, including macrophages[(Boonstra *et al.*, 2006)], dendritic cells[(Boonstra *et al.*, 2006)], eosinophils[(Nakajima *et al.*, 1996; Kayaba *et al.*, 2001)] and, more recently discovered, mast cells, also produce IL-10[(Grimbaldeston *et al.*, 2007)].

### 1.1.3. The IL-10 receptor

IL-10 naturally occurs as a homodimer[(Zdanov *et al.*, 1995)]. The IL-10 receptor, found mainly on haematopoietic cells, is comprised of two subunits, and dimerises into a 4-unit complex upon binding the IL-10 dimer[(Donnelly *et al.*, 1999)]. Formation of the complex allows the kinases JAK1, associated with the IL-10R1 chain, and Tyk2, associated with the IL-10R2 chain, to trans-phosphorylate[(Finbloom and Winestock, 1995; Ho *et al.*, 1995; Kotenko *et al.*, 1997; Riley *et al.*, 1999)]. The now activated kinases transmit the signal downstream via cell-specific pathways (see 1.2.4 and 1.3.4).

## 1.2. T cells and their activation

Figure 1-1 outlines many of the points made in this section.

### 1.2.1. T cell-Antigen Presenting Cell interactions

In addition to MHC-molecule-antigen recognition by the T cell receptor (TCR), effective T cell priming requires costimulation, provided by cognate interaction with molecules on the surface of the antigen-presenting cell (APC). The two key costimulatory molecules are CD80/86 and B7RP-1 which bind to CD28 and Inducible Costimulatory molecule (ICOS), respectively, on the surface of the T cell[(Linsley *et al.*, 1990; Dong *et al.*, 2001)].

CD28 is expressed relatively constantly on T cells, allowing it to receive an early costimulatory signal from CD80/86 on the surface of APC (reviewed in [(Lenschow *et al.*, 1996)]). Binding of CD28 induces tyrosine phosphorylation of the YxxM motif in the cytoplasmic tail by pre-associated kinases[(Raab *et al.*, 1995; Holdorf *et al.*, 1999)]. The phosphorylated motif is a consensus for src-homology-2 (SH2) domains, allowing the subsequent binding of, and signal transduction through, SH2-containing molecules such as phosphatidylinositol 3-kinase (PI3-K)[(Pages *et al.*, 1994; Prasad *et al.*, 1994; Truitt *et al.*, 1994; Ghiotto-Ragueneau *et al.*, 1996)] and growth factor receptor bound protein-2 (Grb-2)[(Kim *et al.*, 1998)].

Costimulation through CD28 is essential for IL-2 production in response to antigen which, in turn, ensures T cell activation as opposed to anergy[(Jenkins *et al.*, 1991; Linsley *et al.*, 1991; Harding *et al.*, 1992; Shahinian *et al.*, 1993)]. Indeed, blockage of CD28 may be sufficient to induce anergy[(Tan *et al.*, 1993)].

ICOS, as its name suggests, exhibits more variable expression: ICOS expression is upregulated following TCR stimulation[(Hutloff *et al.*, 1999; Yoshinaga *et al.*, 1999; McAdam *et al.*, 2000)] and is maintained on experienced T cells[(Coyle *et al.*, 2000; Vieira *et al.*, 2004b)]. ICOS expression can be further enhanced by T cell costimulation via the CD28 pathway described above[(van Berkel *et al.*, 2005)]. Lack of ICOS signalling inhibits T cell activation. This has been demonstrated both by blockade[(Coyle *et al.*, 2000; McAdam *et al.*, 2000; Tesciuba *et al.*, 2001)] and by gene knockout[(Dong *et al.*, 2001; Tafuri *et al.*, 2001)]. Interestingly, both techniques suggested ICOS signalling to be more important for $T_H2$, rather than $T_H1$, responses. However there is also evidence that ICOS is important for $T_H1$ responses[(Guo *et al.*, 2001; Ozkaynak *et al.*, 2001; Sporici *et al.*, 2001)]. ICOS-deficient mice also exhibited limited antibody class-switching; in particular an almost complete lack of IgE[(McAdam *et al.*, 2001)]. The fact that stimulation of CD40 restored class-switching in these mice suggests that ICOS plays a role in T-B cell interactions.

The ligand for ICOS, ICOSL, is expressed on APC and was originally named B7-related protein (B7RP)-1 or B7h because of its homology to the B7 molecules CD80 and CD86 [(Yoshinaga *et al.*, 1999; McAdam *et al.*, 2000; Wang *et al.*, 2000)]. Its expression is more limited than that of CD80 and CD86. One of the cell types on which it is expressed is B cells[(Yoshinaga *et al.*, 1999)]. This adds weight to the theory that ICOS is involved in T-B cell interactions.

In common with CD28, the ICOS cytoplasmic tail has a YxxM motif and is able to recruit, and therefore signal through, PI3-K[(Coyle *et al.*, 2000)]. In contrast to CD28, however, it does not associate with Grb-2[(Coyle *et al.*, 2000)]. This difference may explain why it does not appear to be required for IL-2 production[(Hutloff *et al.*, 1999; Coyle *et al.*, 2000; Harada *et al.*, 2003)]. ICOS does, however, appear to be key in the antibody class-switch to IgE[(McAdam *et al.*, 2001)]. This is relevant for potential treatments for allergic and atopic disorders.

### 1.2.2. DC:T cell clusters and cis-regulation

The key site for T cell–T cell interaction is the DC:T cell cluster. Upon recognising antigen being presented by a lymph node dendritic cell a T cell will interact with the DC for an extended period as it receives stimulatory signals. During that time, other T cells may interact with the same DC due to its presentation of the same or a different antigen. This co-association and the physical proximity of the two (or more) T cells allows molecules expressed by one (presumably the experienced, due to its faster response time) T cell to influence the activation of others. For example if two antigens are presented by the same dendritic cell cytokines produced by experienced, $T_H$-polarised cells specific for one antigen can bias naïve T cells specific for the other[(Creusot *et al.*, 2003a; Schipf *et al.*, 2003)]. In a similar way, regulatory T cells may inhibit the activation of naïve T cells[(Onishi *et al.*, 2008)]. These effects may be mediated either directly or via an effect on the dendritic cell.

A significant consequence of this phenomenon is that an ongoing pathogenic response such as allergy or autoimmunity can enhance similarly inappropriate responses to other, juxtaposed antigens - an effect known as epitope spreading[(Monneaux and Muller, 2002; Klehmet *et al.*, 2004; McMahon *et al.*, 2005)] (reviewed in [(Vanderlugt and Miller, 2002)] and [(Tompkins *et al.*, 2002)]).

### 1.2.3. The plasticity of effector T cell subsets

The paradigm of strict T cell subsets has stood for over two decades. Indeed, the term ‘lineage’ has been used in this context, indicating the commitment by T cells to their surface phenotype and cytokine profile. More recently it has been understood that there is a great deal of plasticity in even polarised effector T cells.

Following many years in which the $T_H1/T_H2$ dichotomy was used to describe effector $CD4^+$ T helper cells, new subtypes of $T_H$ cell have been identified: TH17, TH9[(Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008)], TH22[(Duhen *et al.*, 2009)]. The first of these was the $T_H17$ cell, expressing the RORγt transcription factor and IL-17[(Langrish *et al.*, 2005; Park *et al.*, 2005; Nakae *et al.*, 2007)]. $T_H17$ cells were originally identified as pathogenic in murine EAE and since then have been implicated in several other immune disorders[(Chen *et al.*, 2003b; Cua *et al.*, 2003; Hellings *et al.*, 2003; Murphy *et al.*, 2003; Nakae *et al.*, 2003; Park *et al.*, 2005)].

$T_H17$ cells have been considered to be related to the $T_H1$ subset, in particular because their archetypal cytokines (IL-12 and IL-23, respectively) were shown to share a common subunit[(Aarvak *et al.*, 1999; Oppmann *et al.*, 2000; Parham *et al.*, 2002)]. Other cytokine studies suggested that they are related to regulatory T cells[(Lochner *et al.*, 2008; Zhou *et al.*, 2008)], described in section 1.4. Specifically, TGF-β is associated with the induction of both $T_H17$ and $T_{reg}$ cells[(Bettelli *et al.*, 2006; Veldhoen *et al.*, 2006)]. Despite these observations, there is at least some degree of distinction between T cell subtypes. Antigen experienced cells of one subype are refractory to signals that normally induce another subtype[(Yang *et al.*, 2008b; Guo *et al.*, 2009)] and cytokines which promote one subtype often inhibit the development of another subtype[(Langrish *et al.*, 2005; Kryczek *et al.*, 2007a; Kryczek *et al.*, 2007b)].

Nevertheless, the study of $T_H17$ cells has demonstrated that effector T cells exhibit far more plasticity than was previously appreciated: $T_H17$ cells are able to switch to a $T_H1$ phenotype[(Mathur *et al.*, 2006; Bending *et al.*, 2009; Lee *et al.*, 2009)]. Perhaps more surprisingly, the transformation of $Foxp3^+$ regulatory T cells into inflammatory $T_H17$ cells, has been demonstrated[(Xu *et al.*, 2007; Radhakrishnan *et al.*, 2008; Valmori *et al.*, 2010)]. In humans, IL-17-capable Foxp3+ $T_{reg}$ have been identified, although it is unclear if these cells retain suppressive activity or have an entirely altered phenotype[(Koenen *et al.*, 2008; Beriou *et al.*, 2009; Voo *et al.*, 2009)].

In addition to these studies involving $T_H17$ cells it has come to light that $Foxp3^+$ $T_{reg}$, or at least a proportion of them, can become effector T cells[(Yang *et al.*, 2008a; Duarte *et al.*, 2009; Komatsu *et al.*, 2009; Miyara *et al.*, 2009; Zhou *et al.*, 2009)]. The stability of more recently identified $T_H$ subtypes is also being investigated[(Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008)].

Although the original $T_H1/T_H2$ lineage paradigm clearly stands corrected, it is not yet clear whether all subtypes of effector T cells can change to any other or whether there are fixed preferences and associations between subtypes.

### 1.2.4. The effect of IL-10 on T cell activation

It was established early on that IL-10 inhibits T cell proliferation and IL-2 production[(de Waal Malefyt *et al.*, 1993; Taga *et al.*, 1993)]. As described in 1.2.1 IL-2 synthesis is directly induced by CD28 stimulation. That blockage of this pathway is the mechanism by which IL-10 exerts its effect is confirmed by the inability of IL-10 to inhibit stimulation of T cells by anti-CD3 antibodies – a CD28-independent mechanism[(Del Prete *et al.*, 1993; Akdis *et al.*, 2000)].

It was demonstrated that IL-10 inhibits the phosphorylation of CD28[(Joss *et al.*, 2000)]. In agreement was the observation that, in activated T cells at least, IL-10R associates with CD28[(Akdis *et al.*, 2000)]. Following the binding of IL-10 to IL-10R, trans-phosphorylation of IL-10R-associated JAK1 and Tyk2 (see 1.1.3) allows the association to Tyk2 of SH2-containing protein tyrosine phosphatase 1 (SHP-1)[(Yetter *et al.*, 1995)]. Conformational change in[(Brockdorff *et al.*, 1999; Stefanova *et al.*, 2003; Yang *et al.*, 2003)] and phosphorylation of[(Taylor *et al.*, 2007)] SHP-1, resulting from this association, activate its phosphatase activity. It had been previously demonstrated that SHP-1 can inhibit other PI3 kinase-dependent signalling pathways[(Cuevas *et al.*, 1999)]. The cytoplasmic tail of CD28 contains a YxxM motif which, when phosphorylated, is able to recruit SH2-containing molecules such as PI3 kinase[(Shoelson *et al.*, 1992)]. Later work confirmed that SHP-1,

induced by IL-10, dephosphorylates the cytoplasmic tail of IL-10R-associated CD28, preventing its recruitment of, and signalling through, PI3 kinase[Taylor *et al.*, 2007]. Also featuring YxxM motifs, it is perhaps unsurprising that the IL-10-SHP-1 pathway inhibits costimulation through ICOS and CD2 in a similar way[Taylor *et al.*, 2007; Taylor *et al.*, 2008]. Thus IL-10 inhibits T cell activation through the suppression of costimulatory signal.

In the context of antigenic signal strength, SHP-1 also plays a role in the suppression of the primary TCR signal through ZAP-70[Pani *et al.*, 1996; Plas *et al.*, 1996; Brockdorff *et al.*, 1999; Plas *et al.*, 1999]. This is in apparent contradiction to the earlier observation that anti-CD3-activation of T cells was not inhibited by IL-10[Del Prete *et al.*, 1993; Akdis *et al.*, 2000]. It is possible that SHP-1, in this case, is recruited by a different pathway and not by IL-10.

In addition to the above mechanism, JAK1 and Tyk2 also phosphorylate tyrosine residues within the ITIM domain of the IL-10R cytoplasmic tail[Finbloom and Winestock, 1995; Weber-Nordt *et al.*, 1996]. This allows recruitment of the immunoinhibitory STAT3 transcription factor via association of its SH2 domain with the IL-10R ITIM. This has been shown to induce IL-10 production in T cells[Stumhofer *et al.*, 2007]; thus the local immunosuppressive environment is maintained. The function of STAT3 in T cells has not been studied as extensively as it has in dendritic cells. It will therefore be discussed in more detail in the context of DC (see 1.3.4).

As well as merely limiting the immediate response to antigen, the presence of IL-10 during the activation of T cells has been shown to induce their transformation into regulatory T cells (see 1.4.2). The mechanism by which this is achieved is currently unclear.

## 1.3. Dendritic cells and their maturation

As well as its direct effect, IL-10 also exerts its inhibitory effect on T cells by limiting the efficacy of antigen presenting cells, in particular dendritic cells (DC). Indeed, inhibition via an effect on APC was the mechanism originally described[Bogdan *et al.*, 1991; Fiorentino *et al.*, 1991b; Ding and Shevach, 1992]. Figure 1-1 outlines many of the points made in this section.

### 1.3.1. The maturation model of dendritic cells

Dendritic cells reside in many peripheral tissues where they continuously take up and process antigen. From there they are able to migrate to local lymph nodes and become potent antigen presenting cells. The model describing the transformation from 'immature' antigen-processing cells in the periphery to 'mature' antigen-presenting cells in the lymphoid tissues was formulated over a decade ago[Rescigno *et al.*, 1997; Banchereau and Steinman, 1998] and has since been developed and refined[Banchereau *et al.*, 2000; Guermonprez *et al.*, 2002; Iwasaki, 2007]. Maturation involves, among other things, the upregulation of MHC class II and costimulatory molecules and is generally believed to occur following the receipt of an innate immune stimulus or 'danger signal'. This is often provided via recognition by DC Toll-Like Receptors (TLRs) of Pathogen Associated Molecular Patterns (PAMPs) such as LPS and bacterial DNA or molecules associated with cellular stress or damage such as Heat Shock Proteins (HSPs)[Hagihara *et al.*, 2004; Iwasaki and

Medzhitov, 2004; Tsan and Gao, 2004). Additionally, other cell types, including epithelial cells, B cells and NK cells can mediate DC maturation(Bayry *et al.*, 2005; Munz *et al.*, 2005; Liu *et al.*, 2007).

Dendritic cells are frequently referred to as 'decision makers' or 'conductors' of the immune system (reviewed in (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000)). These names derive from the concept that they direct the immune response appropriately, according to detected danger. The first decision is whether or not a response is initiated: As described above, maturation of DC allows them to become potent antigen-presenting cells. In contrast, under steady-state conditions, when no danger is detected and DC do not mature, they do not induce a response. Much evidence shows that antigen presentation by such immature DC can tolerise T cells (discussed in 1.5). This may provide a system for continuous presentation of, and tolerisation to, self-antigens(Huang *et al.*, 2000).

The second decision can be described as one between qualitatively different types of T cell. In this decision, the nature of the PAMP or other danger signal is key. Stimulation of certain TLRs, such as TLR4 (e.g. by *E. coli* LPS) and TLR9 (e.g. by CpG sequence-rich DNA; see1.7.2) will bias towards $T_H1$ whereas others, such as TLR2 and TLR6 (e.g. by lipopeptides and yeast zymosan) will cause DC to induce a $T_H2$ response(Schnare *et al.*, 2001; Edwards *et al.*, 2002; Dillon *et al.*, 2004) (reviewed in (Zhu *et al.*, 2008b) and (Kaiko *et al.*, 2008)). The subject of TLRs in general is reviewed in (Takeda *et al.*, 2003). The stimuli involved in the differentiation of more recently defined memory T cell subtypes such as $T_H17$ cells(Harrington *et al.*, 2005; Park *et al.*, 2005) and $T_H22$ cells(Duhen *et al.*, 2009; Eyerich *et al.*, 2009) is less well defined. However it does appear that TLR stimulation remains key in the initiation of the differentiation pathway(Martin *et al.*, 2009). Interestingly, recent work on Th17 cells showed TLR expression on T cells and that PAMPs may directly stimulate these cells via these receptors(Reynolds *et al.*, 2010).

### 1.3.2. IL-10 reduces the quantity of antigen presentation

The migration of DC from peripheral to lymphoid tissues is an essential part of their ability to present antigen and activate T cells (reviewed in (Alvarez *et al.*, 2008; Randolph *et al.*, 2008)). IL-10 decreases migration to, and increases emigration from, secondary lymphoid tissues(Wang *et al.*, 1999a; Demangel *et al.*, 2002). This effect appears to be mediated by promoting expression of CCR5, inhibiting expression of CCR7 and prevention of the CCR6 downregulation associated with DC maturation(Dieu-Nosjean *et al.*, 2001; Takayama *et al.*, 2001a).

Evidence also exists that IL-10 can reduce the number of DC and other APC by increasing their rate of spontaneous apoptosis(Ludewig *et al.*, 1995; Raftery *et al.*, 2004; Bailey *et al.*, 2006; Chang *et al.*, 2007).

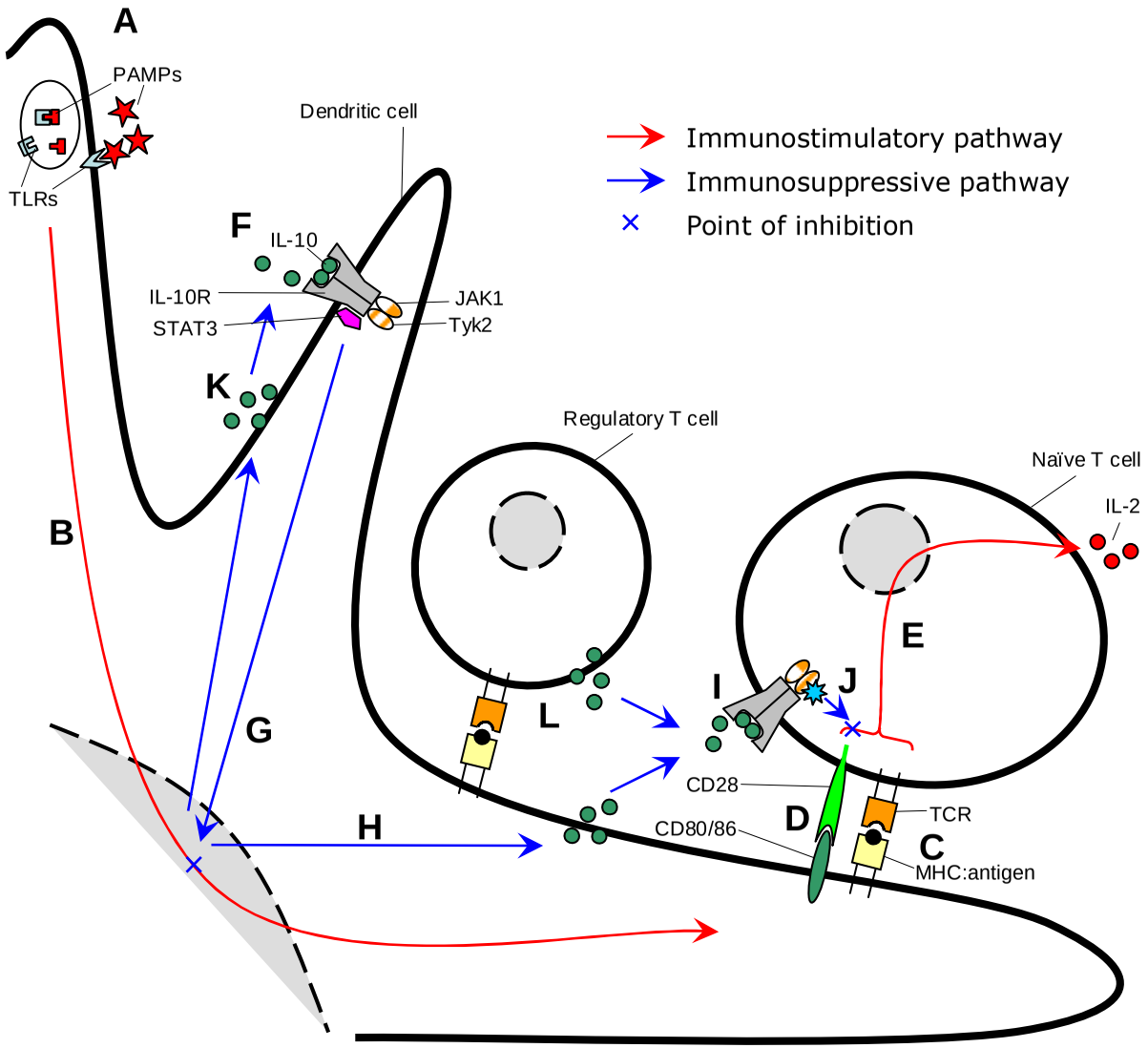



**Figure 1-1. The stimulation of dendritic cells and T cells and the inhibition of both by IL-10.**
The immunostimulatory pathway: A) PAMPs detected by TLR and other molecules on DC cell surface and in intracellular compartments. B) Detection of PAMP induces maturation of DC including upregulation of antigen presenting MHC molecules and costimulatory CD80/86 molecules. C) Recognition of MHC:peptide complex by the TCR. D) Costimulation of T cell via interaction of CD80/86 and CD28. E) Combined TCR+CD28 signal induces T cell activation including, critically, the production of IL-2.
The immunosuppressive pathway: F) IL-10 binds IL-10 receptor, resulting in trans-phosphorylation of JAK1 and Tyk2, phosphorylation of ITIM domain of IL-10R and recruitment and phosphorylation of STAT3. G) STAT3 inhibits the upregulation of genes associated with DC maturation either directly or via the recriutment of other molecules. H) STAT3 also upregulates IL-10 expression by DC. I) IL-10 binds IL-10 receptor (as above), resulting in recruitment and phosphorylation of SHP-1. J) SHP-1 dephosphorylates CD28, preventing its transmission of the costimulatory signal. This directly prevents the production of IL-2 and the activation of the T cell. K) DC-derived IL-10 also acts in an autocrine manner, preserving the immunosuppressive qualities of the DC. L) IL-10-secreting regulatory T cells which recognise antigen on the same DC also provide IL-10 to naive T cells.

### 1.3.3. IL-10 reduces the quality of antigen presentation

Three stimulatory factors are provided by dendritic cells during antigen presentation to T cells: MHC-antigen, costimulatory molecules and cyokines. As described above, the ability of DC to provide these is acquired during maturation. IL-10 has been shown in many different ways to interfere with DC maturation and the ability to efficiently activate T cells.

IL-10 induces MHC class II molecule downregulation by DC[(de Waal Malefyt *et al.*, 1991b; Steinbrink *et al.*, 1997; Faulkner *et al.*, 2000; McBride *et al.*, 2002)]. Alone, this effect, if sufficiently acute, may result in antigenic ignorance in naïve T cells. However it is more likely that a moderate reduction in the number of cell-surface MHC-antigen complexes merely reduces the intensity of the overall stimulatory signal[(Hugues *et al.*, 2004)].

The classical costimulatory molecules, CD80 and CD86, are also downregulated in response to IL-10[(Ding and Shevach, 1992; Ding *et al.*, 1993; Kawamura and Furue, 1995; Mitra *et al.*, 1995)]. Pre-treatment of DC with IL-10 has shown this effect to inhibit the ability of DC to activate naïve T cells[(McBride *et al.*, 2002; Kubsch *et al.*, 2003; Wakkach *et al.*, 2003; Woszczek *et al.*, 2008)]. The inhibitory effect of IL-10 on T cells via suppression of signalling through CD28 was decribed earlier (1.2.4). That CD80/86 are the ligands for CD28 means that IL-10 can inhibit the costimulatory signal via downregulation of both the signal transmitter and the signal receiver. It would be fair to speculate that if IL-10 were present at the immunological synapse these two suppressive mechanisms would likely synergise.

The key cytokine associated with dendritic cell maturation and the ability of DC to activate lymphcytes is IL-12[(Macatonia *et al.*, 1995; Dubois *et al.*, 1998; Berberich *et al.*, 2003; Mandrekar *et al.*, 2004)]. There is evidence that, in this way, IL-12 is more important for the induction of $T_H1$ responses[(Macatonia *et al.*, 1995; Berberich *et al.*, 2003)]. However, as well as having a direct effect on lymphocytes, autocrine IL-12 is also important for the complete maturation of DC[(Hilkens *et al.*, 1997; Bianchi *et al.*, 1999)]. In this way, DC-produced IL-12 is vital to the development of T cell responses in general.

IL-10 has long been known to downregulate IL-12 production by DC[(De Smedt *et al.*, 1997)]. Indeed, the presence of IL-10 inhibits IL-12 production [(Takayama *et al.*, 1998; Xia and Kao, 2002; Xia and Kao, 2003)] and its absence allows DC to mature and present antigen more effectively[(Demangel *et al.*, 2002)]. The inverse is also true: Neutralisation of IL-12 *in vivo* can result in the induction of tolerance[(Riemann *et al.*, 1996)]. It has been suggested that the mechanism of this reciprocal relationship is at the transcriptional level[(Aste-Amezaga *et al.*, 1998)]. However the blocking of NF-κB also appears to be involved[(Wang *et al.*, 1995; Bhattacharyya *et al.*, 2004)]. Interestingly, there is evidence that, whilst it does inhibit IL-12 production, IL-10 still allows transient maturation of DC and that this may give rise to tolerogenic DC[(Perona-Wright *et al.*, 2007)] (see section 1.5).

Naturally occurring, IL-10-producing DC have been identified[(Akbari *et al.*, 2001; Corinti *et al.*, 2001; Monteleone *et al.*, 2008)]. That autocrine IL-10 might allow these cells to maintain their $IL\text{-}10^{+}IL\text{-}12^{-}$ phenotype and that autocrine IL-12 allows activated DC to reach full maturity promotes the idea that a critical decision is made at or around the time of activation, after which time DC are either IL-12-producers or IL-10-producers[(Jiang *et al.*, 2002; Wakkach *et al.*, 2003)]. However, mutually exclusive production of these two cytokines is not necessarily always true; a DC population has been identified that produces both IL-10 and IL-12[(Muthana *et al.*, 2006)]. Nevertheless, that IL-10 inhibits DC production of IL-12 is clear.

### 1.3.4. The mechanism of the effect of IL-10 on antigen presenting cells

As described above (see 1.1.3 and 1.2.4), binding of the IL-10 dimer by the IL-10 receptor results in trans-phosphorylation and activation of JAK1 and Tyk2 as well as phosphorylation of tyrosine residues within the ITIM domain of the IL-10R cytoplasmic tail. This allows recruitment of the immunoinhibitory STAT3 transcription factor via association of its SH2 domain with the IL-10R ITIM.

The antiinflammatory effects of IL-10 on APC are mediated entirely through the STAT3 transcription factor. In mice, disruption of the STAT3 gene in macrophages and neutrophils causes constitutive activation and nonresponsiveness to IL-10 in these cells and the development of colitis in the animal(Takeda *et al.*, 1999). Microarray experiments identify many transcriptional changes induced by IL-10; reexamination in STAT3-deficient cells showed all these changes to be dependent on STAT3(Lang *et al.*, 2002a). Transfection of macrophages with a dominant negative STAT3 showed similar effects(Williams *et al.*, 2004). In contrast, constitutively active STAT3 can emulate exposure to IL-10(Williams *et al.*, 2007), as can the artificial engineering of other receptors to activate STAT3(El Kasmi *et al.*, 2006). In a similar way to viral homologues of IL-10 (1.1.1), the importance of STAT3 in the suppression of the inflammatory response is demonstrated by a pathogen: Toxoplasma gondii directly, and independently of IL-10, activates STAT3, suppressing the potential inflammatory response that would protect the host(Butcher *et al.*, 2005; Saeij *et al.*, 2007).

Downstream of STAT3, the IL-10-induced antiinflammatory pathway is still relatively unclear. However, it appears to be mainly due to inhibition of transcription of genes normally upregulated by detection of PAMPs by TLRs(Lang *et al.*, 2002a; Murray, 2005). Several molecules have been suggested as targets for STAT3 including suppressor of cytokine signalling (SOCS)3(Berlato *et al.*, 2002; Lang *et al.*, 2003; Li *et al.*, 2006), BCL3(Kuwata *et al.*, 2003; Wessells *et al.*, 2004; Riemann *et al.*, 2005), ETV3 and SBNO2(El Kasmi *et al.*, 2007).

## 1.4. Regulatory T cells

Healthy animals and humans contain potentially self-reactive or otherwise pathogenic T cells that escape deletion during their thymic development(Fowell and Mason, 1993; Hafler and Weiner, 1995; Asseman *et al.*, 2003). Nevertheless, autoimmunity and allergy do not occur in the majority of individuals. “Suppressor” T cells were first proposed as a protective mechanism over 30 years ago(Gershon and Kondo, 1970; Gershon and Kondo, 1971; McCullagh, 1973). However, amongst other things, a lack of tools led to the evolution of increasingly complex models and explanations. As technologies advanced, particularly those of genetic sequencing and monoclonal antibodies, many highly-regarded theories did not stand up to scrutiny and the field as a whole became tainted. Only years later was the subject revisited in a series of experiments that culminated in proof that ‘infectious’ tolerance was mediated by $CD4^+$ T cells(Boitard *et al.*, 1989; Qin *et al.*, 1990; Qin *et al.*, 1993).

That tolerance is maintained by one or more subsets of T cells whilst immunity, both protective and pathogenic, is mediated by others was suggested by several different studies. Thymectomy or irradiation-induced lymphopoenia can lead to autoimmunity(Penhale *et al.*, 1975) whilst adoptive transfer of

normal lymphocytes can prevent this[Penhale *et al.*, 1976]. Protective T cells, albeit poorly defined, could be isolated from untreated animals at an early stage[Mordes *et al.*, 1987; Boitard *et al.*, 1989]. An early distinction between pathogenic and protective T cells was provided by the induction of pathogenic immunity by transfer of naïve, $CD45RB^{HI}$ T cells into SCID mice and protection from this disease by cotransfer of antigen-experienced, $CD45RB^{LO}$ T cells[Powrie *et al.*, 1993]. Importantly, it was observed that the same subgroups were also responsible for the induction and prevention of protective immunity in a pathogen model[Powrie *et al.*, 1994].

### 1.4.1. Naturally occurring $T_{reg}$ cells

To efficiently study regulatory T cells it was necessary to identify a phenotypic marker more unique than CD45RB. CD25 was shown to be a suitable marker when depletion of $CD25^+$ cells from lymphocyte suspensions adoptively transferred into athymic nude mice resulted in autoimmunity[Sakaguchi *et al.*, 1995]. *In vitro* studies confirmed that $CD4^+CD25^+$ T cells suppress T cell responses to antigen and showed that they require recognition of antigen to function in this way and that the mechanism of suppression is cell contact-dependent[Thornton and Shevach, 1998; Stephens *et al.*, 2001]. Identification of $CD4^+CD25^+$ regulatory T cells in human blood provided further confirmation of the nature of these cells and of the validity of CD25 as a reliable marker[Dieckmann *et al.*, 2001; Jonuleit *et al.*, 2001].

CD25 is the IL-2 receptor α chain and thus widely expressed on T cells, especially during their activation. Therefore its use as an identifier of $T_{reg}$ has always been limited. Additionally, potential blocking of the IL-2 receptor is a caveat in experiments that enrich the $CD25^+$ population using antibodies. A more suitable identifier was identified following genetic examination of autoimmune-prone scurfy mice and humans suffering from immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX); a mutation was discovered in the gene for the forkhead transcription factor Foxp3[Bennett *et al.*, 2001; Brunkow *et al.*, 2001; Wildin *et al.*, 2001]. High expression of this gene was discovered in $CD4^+CD25^+$ $T_{reg}$ [Fontenot *et al.*, 2003; Hori *et al.*, 2003; Khattri *et al.*, 2003]. Later, its expression was shown to correlate with suppressor activity even in the absence of CD25 expression[Fontenot *et al.*, 2005].

Development of a bicistronic reporter system has allowed the identification of $Foxp3^+$ cells *ex vivo*[Wan and Flavell, 2005]. Nevertheless, Foxp3 is not merely a conventient marker for immunologists. It is required for regulatory activity of naturally-occurring $T_{reg}$[Gavin *et al.*, 2007] and artificially induced expression of Foxp3 can actually invoke regulatory properties in T cells[Fontenot *et al.*, 2003; Hori *et al.*, 2003].

Not all CD4+CD25+ $T_{reg}$ characteristics are directly controlled by Foxp3[Sugimoto *et al.*, 2006; Gavin *et al.*, 2007]. It is becoming clear that the decision to become a $T_{reg}$ involves a mechanism upstream of Foxp3[Gavin *et al.*, 2007; Hill *et al.*, 2007]. In fact, there is evidence that $T_{reg}$ can develop in the absence of Foxp3[Lin *et al.*, 2007]. Downstream, Foxp3 appears to prevent expression of key genes involved in T cell activation[Li *et al.*, 2007; Marson *et al.*, 2007].

Whilst early literature suggested that regulatory T cells were anergic, traditional T cell stimuli such as mature dendritic cells and IL-2 are able to induce proliferation of $CD4^+CD25^+$ T cells[Levings *et al.*, 2001; Yamazaki *et al.*, 2003; Yamazaki *et al.*, 2006]. In fact, it appears that IL-2 is actually required for the expansion and function of these cells[Furtado *et al.*, 2002; Thornton *et al.*, 2004; Setoguchi *et al.*, 2005].

Suppression of T cell responses by $CD4^+CD25^+$ $T_{reg}$ is antigen-specific. One possible mechanism is competition for cytokines and presented antigen[(de la Rosa *et al.*, 2004; Barthlott *et al.*, 2005; Brandenburg *et al.*, 2008; Onishi *et al.*, 2008)]. In addition, however, their antigen-specific activation still allows their suppressive effects to be mediated upon any neighbouring T cells, likely via downregulation of APC costimulatory molecules[(Cederbom *et al.*, 2000; Misra *et al.*, 2004; Lewkowich *et al.*, 2005; Oderup *et al.*, 2006; Onishi *et al.*, 2008)]. Adoptive transfer studies with multiple transgenic T cells and their corresponding peptide antigens have shown that this mechanism allows $CD4^+CD25^+$ $T_{reg}$ to suppress a response to an unrelated, but proximally close, antigen[(Takahashi *et al.*, 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000; Tanchot *et al.*, 2004; Sanchez-Fueyo *et al.*, 2006)].

### 1.4.2. Inducible $T_R1$ cells

IL-10-secreting regulatory T cells were first proposed following HLA-mismatched stem cell transplants that did not result in graft-versus-host disease[(Bacchetta *et al.*, 1994)]. *In vitro*-generated IL-10-secreting regulatory T cells were subsequently shown to suppress pathogenic immunity[(Groux *et al.*, 1997)]. It is now understood that these cells also protect against pathogenic immune responses to autoantigens[(Kitani *et al.*, 2000)], commensal bacteria[(Duchmann *et al.*, 1995; Cong *et al.*, 2002)] and allergens[(Akdis *et al.*, 2004)].

It appears that a major role of these $T_R1$ cells is the mediation of tolerance at mucosal and skin sites where they prevent pathogenic immunity to foreign, nonpathogenic antigens. Both the discovery of $T_R1$ cells and the bulk of later work are focused on these tissues[(Powrie *et al.*, 1993; Groux *et al.*, 1997; Cong *et al.*, 2002)]. It is likely that the generation of $T_R1$ cells also occurs at these sites[(Duchmann *et al.*, 1995; Meiler *et al.*, 2008)].

The importance of IL-10 for the regulatory activity of $T_R1$ cells is demonstrated by the inability of $CD45RB^{LO}$ cells from $IL\text{-}10^{-/-}$ mice to protect against colitis induced by $CD45RB^{HI}$ cells (see 1.4)[(Asseman *et al.*, 1999)] and by the ability to artificially generate regulatory T cells that will protect against colitis by transfection of T cells with the IL-10 gene[(Van Montfrans *et al.*, 2002a; Van Montfrans *et al.*, 2002b)]. Mice in which the IL-10 gene has been disrupted in T cells only suffer from colitis and other syptoms in a similar way to mice in which the gene has been disrupted in all cells[(Roers *et al.*, 2004)]. Further restriction of this inactivation to $Foxp3^+$ T cells results in much less severe disease, highlighting the distinction between the $Foxp3^+$ and $T_R1$ subsets of $T_{reg}$[(Rubtsov *et al.*, 2008)].

Artificial antigenic stimulation of $CD4^+$ T cells in the presence of IL-10 induces the generation of $T_R1$ cells[(Groux *et al.*, 1996; Groux *et al.*, 1997; Cong *et al.*, 2002)] as do antigen presentation by IL-10-treated dendritic cells (see 1.5.2), antigen presentation by immature DC (see 1.5.1) and specific immunotherapy[(Meiler *et al.*, 2008)]. These phenomena are dependent on IL-10 and it is likely that they reflect, to some degree, natural antigen presentation under steady-state, noninflammatory conditions. Therefore IL-10 also plays a key role in the generation of $T_R1$ cells.

More recently it was observed that $T_R1$ cells can also be induced by naturally-occurring $Foxp3^+$ $T_{reg}$[(Dieckmann *et al.*, 2002; Jonuleit *et al.*, 2002; Kearley *et al.*, 2005; Wakkach *et al.*, 2008)]. $Foxp3^+$ $T_{reg}$ are certainly not always required for $T_R1$ induction[(Levings *et al.*, 2004)] and even when this is the mechanism responsible for their generation direct suppressive activity by the $Foxp3^+$ cell is maintained[(Jonuleit *et al.*, 2002)]. Nevertheless, these studies demonstrate the interrelationship between subsets of regulatory T cell.

Similarly to Foxp3$^+$ $T_{reg}$ $T_R1$ cells are activated in an antigen-specific manner[(Bacchetta *et al.*, 1994; Groux *et al.*, 1997; Cong *et al.*, 2002; Yu *et al.*, 2005)]. A particularly interesting example of this is the normal tolerance of intestinal leukocytes to self gut flora versus their strong proliferation in response to such bacteria from the intestine of another individual[(Duchmann *et al.*, 1995)]. Whilst their activation may be antigen-specific, the suppressive action of $T_R1$ cells is mediated by a secreted cytokine and therefore precludes a purely antigen-specific action on responding T cells. Paracrine effects of IL-10 can inhibit the activation of any juxtaposed T cells within a DC cluster. Therefore, whilst suppression may be largely antigen-specific, regulation of responses to other antigens being simultaneously presented is also likely.

### 1.4.3. The distinction between regulatory T cell subsets is becoming blurred

Whilst there are certainly differences in the origins and functions of the regulatory T cell subsets the traditional division between naturally occurring CD25$^+$, Foxp3$^+$ $T_{reg}$ and inducible IL-10-secreting $T_{reg}$ has become somewhat less distinct. Perhaps the best example of this is the identification of IL-10-secreting CD4$^+$CD25$^+$ $T_{reg}$ and the recognition of their importance in protection from disease[(Akdis *et al.*, 1998; Annacker *et al.*, 2001; Francis *et al.*, 2003; McGeachy *et al.*, 2005; Yu *et al.*, 2005; Uhlig *et al.*, 2006; Maynard *et al.*, 2007)].

Whilst it was believed that the Foxp3 transcription factor defined a separate lineage of regulatory T cell, it has been shown that IL-10-secreting $T_{reg}$ can derive both from Foxp3$^-$ and from Foxp3$^+$ thymocytes[(Maynard *et al.*, 2007)]. A newly identified marker, TIRC7, is found on both CD25$^+$ and IL-10-secreting $T_{reg}$ and may distinguish a subset of CD25$^+$ $T_{reg}$ that have the potential to become IL-10-secreting $T_R1$ cells[(Wakkach *et al.*, 2008)].

The origin of CD25$^+$Foxp3$^+$ $T_{reg}$ has also been questioned: Foxp3$^+$ $T_{reg}$ derived from Foxp3$^-$ thymocytes have been observed[(Maynard *et al.*, 2007)]. Similarly, the characterisation of these cells as 'naturally occurring', as opposed to inducible, is contested by work showing at least some degree of inducibility, either by *in vitro* antigen presentation in the presence of TGF-β[(Chen *et al.*, 2003a; Fantini *et al.*, 2004)] or by tolerogenic antigen administration protocols[(Thorstenson and Khoruts, 2001; Zhang *et al.*, 2001; Hauet-Broere *et al.*, 2003; Kretschmer *et al.*, 2005)]. Under natural conditions, TGF-β produced by existing Foxp3$^+$ $T_{reg}$ or by DC could induce naïve CD4$^+$ T cells to become $T_{reg}$[(Luo *et al.*, 2007; Andersson *et al.*, 2008)].

In other experiments, IL-10-secreting $T_{reg}$ can show similar properties to CD4$^+$CD25$^+$ $T_{reg}$ including IL-10-independent suppression[(Vieira *et al.*, 2004a)]. A caveat to this observation is that these IL-10$^+$ $T_{reg}$ were induced *in vitro* with a combination of vitamin D3 and dexamethasone, as opposed to the more traditional treatment with IL-10, and have a slightly different cytokine profile[(Barrat *et al.*, 2002)].

Despite these observations, the existing division of $T_{reg}$ subtypes is likely to be appropriate to some degree. The isolation of viable IL-10-producing T cells based solely on this feature and without a unique cell surface marker is difficult. Therefore studies on pure populations have not been carried out. Whilst Foxp3$^+$ cell-derived IL-10 appears to play a role it is not the only IL-10-dependent mechanism of suppression by these cells; their induction of $T_R1$ cells is probably still more important[(Maynard *et al.*, 2007)]. For these reasons it has been suggested that $T_R1$ cells are more strictly defined as those whose induction and regulatory activity are dependent on IL-10[(Maynard and Weaver, 2008)].

Finally, the overall function of $T_{reg}$ subtypes may be more relevant than their detailed phenotype and origin. In particular, and following on from initial studies, it appears that the key function of IL-10-secreting $T_R1$ cells is the regulation of immune responses at mucosal interfaces[(Uhlig *et al.*, 2006; Rubtsov *et al.*, 2008)]. It has been suggested that these cells regulate activated T cells which have already reached inflamed tissue whilst $Foxp3^+CD25^+$ $T_{reg}$ act upstream, inhibiting activation in the secondary lymphoid tissues[(Samy *et al.*, 2005)] (reviewed in [(Maynard and Weaver, 2008)]).

## 1.5. The role of DC in induction of regulatory T cells

### 1.5.1. Immature DC as inducers of tolerance

As mentioned in 1.3.1, DC are key in the decision between immunity and tolerance. Perhaps the most widely accepted theory regarding this is that antigen presentation by mature DC (mDC) induces immunity whilst that by immature DC (iDC) induces tolerance. An attempt to develop an effective immunisation protocol supported this theory when it was discovered that targeting antigen to DC which have received no maturation stimulus resulted in tolerance, rather than immunity[(Hawiger *et al.*, 2001; Bonifaz *et al.*, 2002; Mahnke *et al.*, 2003; Probst *et al.*, 2003)].

*In vitro*, stimulation with allogeneic immature DC induced naïve T cells to become regulatory T cells, specifically IL-10-producing $T_R1$ cells[(Jonuleit *et al.*, 2000; Levings *et al.*, 2004)]. The importance of the immaturity of the DC is reinforced by studies which target antigen to DC whilst simultaneously providing a DC maturation signal[(Bonifaz *et al.*, 2002; Bonifaz *et al.*, 2004)]. *In vivo*, targeting of antigen to iDC and administration of antigen-pulsed iDC both resulted in tolerisation to antigen[(Dhodapkar *et al.*, 2001; Bruder *et al.*, 2005)].

The immune system is constantly exposed to innocuous foreign antigens such as those in food and pollens. It is generally understood that, due to the normal absence of inflammation (sometimes referred to as steady-state conditions), these antigens are presented by immature dendritic cells, thus inducing or maintaining tolerance. It is likely that the tolerogenic effect of certain immunotherapeutic antigen administration protocols, such as oral and nasal tolerisation and specific immunotherapy, is mediated by this same mechanism[(Chen *et al.*, 1994; Akdis and Blaser, 1999; Akbari *et al.*, 2001; Hurst *et al.*, 2001; Thorstenson and Khoruts, 2001; Hauet-Broere *et al.*, 2003; Unger *et al.*, 2003; Ostroukhova *et al.*, 2004; Van Hove *et al.*, 2007)].

### 1.5.2. IL-10-treated DC and IL-10-producing DC as inducers of tolerance

The above tolerisation of T cells by immature DC, including the technique of Specific Immunotherapy (SIT), appears to be dependent on DC-derived IL-10[(Akbari *et al.*, 2001; Levings *et al.*, 2004)] and can be enhanced by IL-10-inducing adjuvants[(Van Overtvelt *et al.*, 2008)]. *In vivo* studies show that IL-10-expressing DC naturally occur in mucosal tissues such as the lung[(Akbari *et al.*, 2001)] and gut[(Chirdo *et al.*, 2005)] as well as immune tissues[(Wakkach *et al.*, 2003)] and that they tolerise T cells to antigen.

A caveat to the distinction being drawn here is that even if immature DC do induce tolerance, in certain situations, DC maturation may not prevent, and might even be a requirement for, tolerisation[(Lundqvist *et al.*, 2005; Lau *et al.*, 2008)]. Nevertheless, there is evidence that autocrine IL-10 prevents

*full* maturation of DC; it could be argued that only full maturation leads to immune stimulation[(Corinti *et al.*, 2001; Demangel *et al.*, 2002; Monteleone *et al.*, 2008)].

Autocrine regulation of DC by IL-10 can be studied by artificial exposure of DC to the cytokine. Pretreatment of DC with IL-10 has been repeatedly shown to confer tolerogenic properties, including the secretion of IL-10 itself[(Steinbrink *et al.*, 1997; Steinbrink *et al.*, 1999; Muller *et al.*, 2002; Steinbrink *et al.*, 2002; Kubsch *et al.*, 2003; Wakkach *et al.*, 2003; Li *et al.*, 2009)]. Indeed, the STAT3 signalling pathway induced by IL-10 induces upregulation of the same cytokine, forming a positive feedback loop[(Benkhart *et al.*, 2000; Ziegler-Heitbrock *et al.*, 2003; Staples *et al.*, 2007)]. Antigen presentation by these DC to naïve T cells renders the T cell anergic, possibly itself becoming a regulatory T cell [(McBride *et al.*, 2002; Fu *et al.*, 2008; Yamaura *et al.*, 2008)]. Interestingly, whilst suppressing the adaptive immune response, IL-10-treated DC show an intact, or even enhanced, ability to contribute towards the innate, inflammatory response[(Nolan *et al.*, 2004)].

Although the tolerogenic properties of immature DC appear to be dependent on their production of IL-10[(Koya *et al.*, 2007)], the inverse is not necessarily true: Naturally-occurring, IL-10-secreting, tolerogenic DC that are phenotypically mature have been described[(Akbari *et al.*, 2001; Akbari *et al.*, 2002)]. Finer dissection may explain these different findings; altered kinetics[(Perona-Wright *et al.*, 2007)] and reduced IL-12 expression (irrespective of surface phenotype; see section 1.3.3) are two existing theories.

Autocrine IL-10 feedback may be a key to DC-induced tolerance. Indeed, transgenic mice expressing IL-10 via the MHC class II promoter are highly resistant to induction of autoimmunity[(Cua *et al.*, 1999)]. Studies of IL-10-transfected DC have demonstrated greatly inhibited T cell stimulatory activity[(Takayama *et al.*, 1998; Takayama *et al.*, 1999)] and an ability, at least under certain conditions, to induce $T_R1$ regulatory T cells[(Fu *et al.*, 2008)]. Both the pre-treatment and the transfection approaches have been extended into treatment of immune disorders (discussed in sections 1.6.3 and 1.6.4).

### 1.5.3. Specialised subsets of DC?

As central controllers, DC may select between immunity and tolerance. However, there is evidence that specialised tolerising DC may exist [(Akbari *et al.*, 2001; Iwasaki and Kelsall, 2001; Chirdo *et al.*, 2005; Yamazaki *et al.*, 2008)]. Most evidence comes from studies on intestine; it is perhaps logical that the mucosal tissue most constantly and intensively in contact with foreign antigen be that which most requires a rigorous tolerisation mechanism[(Coombes *et al.*, 2007; Sun *et al.*, 2007)]. As discussed in 1.4.3, it appears that TGF-β plays a key role in the induction of $Foxp3^+$ $T_{reg}$ by intestinal DC[(Coombes *et al.*, 2007; Luo *et al.*, 2007)].

The existence of these tolerising DC may not contradict the paradigm of the DC as a decision-maker. That they tend to be located in a specific tissue suggests that their tolerising characteristics such as TGF-β expression and even expression of phenotypic markers such as CD103[(Coombes *et al.*, 2007; Sun *et al.*, 2007)] may be a response to site-specific stimuli in the in gut[(Ginhoux *et al.*, 2009)] rather than indicating a distinct lineage. Indeed, there is evidence that factors secreted by intestinal epithelial cells are able to induce these characteristics in monocyte-derived DC[(Iliev *et al.*, 2009)]. Nevertheless it appears that such tissue-specific effects may remain even if the DC is removed from its original environment[(Akbari *et al.*,

2001)</sup>. Therefore even if tolerogenic DC are a product of their environment they have the potential to remain, to some degree, specialised.

## 1.6. IL-10 used against disease

The ever-strengthening connection between IL-10 and immune suppression/tolerance has led to many investigations into the therapeutic use of the cytokine against immune diseases (reviewed in (O'Garra *et al.*, 2008)). Technological and scientific advances have led to the use of increasingly sophisticated methods of cytokine administration, allowing increased accuracy and specificity. It appears clear that no single treatment is suitable for all disorders; this in itself provides a better understanding of the variety of pathological mechanisms involved.

### 1.6.1. Recombinant IL-10

Experimental Autoimmune Encephalomyelitis (EAE) is a well defined model of human multiple sclerosis. The EAE autoantigen is myelin basic protein (MBP), against which transgenic T cells have been developed. It was demonstrated early on that rats subcutaneously injected with IL-10 during and after immunisation with MBP were resistant to EAE induction(Rott *et al.*, 1994). Intranasal administration of IL-10 was also shown to be effective(Xiao *et al.*, 1998). Interestingly, intravenous administration of IL-10 was unable to inhibit the development of EAE(Cannella *et al.*, 1996). This suggests that the route and timing of IL-10 administration are important for its immunosuppressive properties. That the IL-10 receptor is expressed on a wide variety of cells and that its effects can be widespread would agree that, indeed, targeting delivery to a specific location or cell type might be desirable.

Orally delivered, polymer-coated gelatin microparticles containing recombinant IL-10 have been used to target the cytokine to the large intestine(Bhavsar and Amiji, 2008). This technique improved the histological and clinical outcome in studies on a model of colitis. An IL-10-IL-1 receptor agonist fusion protein was developed to target inflamed joint synovia in a model of rheumatoid arthritis (Chang *et al.*, 2004a).

Recombinant IL-10 has been tested in clinical trials against psoriasis(Asadullah *et al.*, 2001; Reich *et al.*, 2001) and Crohn's disease(Tilg *et al.*, 2002) as well as against febrile responses associated with expermental endotoxaemia(Pajkrt *et al.*, 1997). In general, treatments were able to inhibit certain cytokines and symptoms but side effects included upregulation of other cytokines and flu-like symptoms. These studies demonstrate the broad spectrum of IL-10's activity as both an immune suppressor and stimulator. It also illustrates a significant limitation of the systemic administration of normally locally-produced, short-lived cytokines.

### 1.6.2. IL-10-expressing bacteria

That the site of IL-10 administration is important in the inhibition of immune disorders makes diseases of specific, accessible organs attractive for treatment. As described in 1.4.2, IL-10-induced, IL-10-secreting $T_R1$ cells were first defined in the study of colitis. Intestinal immune disorders are therefore obvious candidates for IL-10 treatment.

Inflammatory bowel disorders are often caused by undesired immune responses to non-pathogenic, commensal microorganisms[(Singh *et al.*, 2001)]. As described in 1.1.1, IL-10 has been shown to be important in preventing these disorders. A novel approach was developed whereby a gut-commensal lactococcus species was genetically engineered to express IL-10[(Steidler *et al.*, 2000)]. Oral administration resulted in a significant inhibition of colitis. The bacterium was further engineered to be dependent on thymine and thymidine, ensuring it would not be viable in the environment[(Steidler *et al.*, 2003)]. Several technological steps were made to increase the efficiency of administration[(Huyghebaert *et al.*, 2005a; Huyghebaert *et al.*, 2005b; Termont *et al.*, 2006)]. A phase I clinical trial demonstrated that this is a promising method of combating an immune disorder with IL-10[(Braat *et al.*, 2006)].

The engineered bacterium appears to penetrate into the mucosa[(Waeytens *et al.*, 2008)]. *In vitro* work suggests that at least one mechanism of action is the modulation of DC maturation[(Loos *et al.*, 2009)].

### 1.6.3. IL-10-treated DC

As described earlier (see 1.5.2) *in vitro* IL-10 treatment of DC can confer tolerogenic properties. This phenomenon was established by studying the interaction of these DC with T cells *in vitro*. Reduced stimulatory ability of these DC has also been shown upon their return to the body[(Haase *et al.*, 2002)]. The possibility of using IL-10-treated DC to induce tolerance in disease situations has been explored. In mice, such DC were able to inhibit airway hyperresponsiveness in an ovalbumin allergy model[(Koya *et al.*, 2007)] and to prevent weight loss and histopathology in a SCID model of colitis[(Pedersen *et al.*, 2007)]. The facilitation of antigen presentation by the treated DC (by pulsing with OVA or enterobacterial extract, respectively) was important in both cases. IL-10-treated DC have also been shown to be effective against collagen-induced arthritis (CIA)[(Kim *et al.*, 2005)] and experimental myasthenia gravis[(Xiao *et al.*, 2006)]. The latter study was extended into the clinical setting with some success.

### 1.6.4. IL-10-transfected DC

As described in 1.5, IL-10 expression appears to be more important in determining the immunogenicity vs tolerogenicity of a DC than do maturation-specific cell surface phenotypic markers. Several studies have examined the forced expression of IL-10 by antigen-presenting DC as a way to induce antigen-specific tolerance.

*In vitro* data shows that transfection of DC with IL-10-encoding retrovirus[(Takayama *et al.*, 1998)] or adenovirus[(Whalen *et al.*, 2001; Rea *et al.*, 2004)] inhibits their ability to stimulate T cells. The authors suggest that these cells could potentially be used to treat immune disease. This possibility is expanded by a refinement; the inclusion of the eGFP gene, allowing potentially tolerogenic cells to be purified for use in treatment of disease[(Takayama *et al.*, 1999)].

Transfer of IL-10-transfected DC to experimental animals has been demonstrated to protect against several immune pathogeneses. Lipid, retroviral and adenoviral vectors have been successfully employed in this manner against asthma[(Henry *et al.*, 2008)], allograft rejection[(Coates *et al.*, 2001; Moore *et al.*, 2004; Zhang *et al.*, 2004; Zhu *et al.*, 2008a)], colitis[(Van Montfrans *et al.*, 2002a)], myocarditis[(Yang *et al.*, 2006)] and hyperimmune responses such as DTH[(Whalen *et al.*, 2001)] and septic shock[(Oberholzer *et al.*, 2001)]. Nevertheless, it is clear that effective

suppression by IL-10-transfected DC *in vitro* does not automatically translate into success *in vivo*(Buonocore *et al.*, 2002).

Similarly to *ex vivo* treatment of DC with IL-10, although these attempts to treat disease with IL-10-transfected DC have shown some success, approaches involving ex vivo treatment of cells followed by their return to the body are complex: They must take into account histocompatibility and/or involve patient-tailored treatments. Transfection *in vivo* overcomes these problems.

The use of retroviral and adenoviral vectors to transfer the IL-10 gene has been extended *in vivo*. Similarly to the above *ex vivo* work, some inhibition of asthma(Stampfli *et al.*, 1999), DTH(Whalen *et al.*, 2001), septic shock(Oberholzer *et al.*, 2001; Oberholzer *et al.*, 2002; Oberholzer *et al.*, 2005), EAE(Cua *et al.*, 2001) and CIA(Apparailly *et al.*, 1998) has been documented. Interestingly, whilst an IL-10-encoding viral vector has been shown to protect against destruction of synergeic islet transplants due to preexisting immunity to islet antigen(Zhang *et al.*, 2003), it was not able to prevent rejection of islet allotransplants(Zhang *et al.*, 2005). It is important to note that, as opposed to viral transfection of DC *ex vivo*, viral vectors administered *in vivo* may transduce multiple cell types. Nevertheless there is evidence that some such vectors do exhibit a degree of specificity to DC(Oberholzer *et al.*, 2002; Oberholzer *et al.*, 2005).

Sections 1.5.1 and 1.5.2 discuss the immature-tolerogenic / mature-immunogenic model of DC and that IL-10 expression by DC may be more important than their maturation state. There is evidence that retroviral vectors have inhibitory effects on DC maturation and stimulatory ability, independent of any transgene(Chen *et al.*, 2004). In contrast, recombinant adenovirus alone has been shown to mature DC(Morelli *et al.*, 2000; Oberholzer *et al.*, 2002). That adenovirus vectors have shown a degree of success in IL-10-induced immune regulation agrees with the above notion that an immature state is not critical for tolerising: perhaps mature (or semi-mature) DC expressing IL-10 might even be more effective(Rea *et al.*, 2004).

The outcome of administration of IL-10-treated or –transfected DC, or of IL-10-encoding viral vector appears to be dependent on the route of administration. IL-10-treated DC successfully inhibited disease when administered to the peritoneum but did not when injected subcutaneously(Xiao *et al.*, 2006; Pedersen *et al.*, 2007). IL-10-transfected DC induced allograft tolerance when injected into the portal vein but not the tail vein(Zhang *et al.*, 2004). IL-10-expressing adenovirus improved the survival rate in a murine sepsis model when injected directly into the thymus(Oberholzer *et al.*, 2001) and prevented the induction of EAE when injected directly into the CNS(Cua *et al.*, 2001). In both cases, peripheral administration was ineffective.

## 1.7. Genetic immunisation

The administration of DNA encoding antigen in order to elicit an immune response to the expressed protein was first proposed almost 20 years ago(Tang *et al.*, 1992). Quickly several groups produced data supporting this suggestion(Robinson *et al.*, 1993; Ulmer *et al.*, 1993; Wang *et al.*, 1993) and protection against infectious disease was demonstrated(Lai *et al.*, 1995; Ulmer *et al.*, 1998; Walker *et al.*, 1998; Cornell *et al.*, 1999; Weiss *et al.*, 2000). Genetic immunisation has advantages over traditional protein-based vaccines at both the design and the administration stages. Firstly, it is possible to rapidly test the genome of a pathogen for immunogeneic sequences/epitopes(Haddad *et al.*, 2004). The process does not involve complex protein purification steps and,

in the case of naked DNA vaccines, does not carry the risks of using attenuated pathogens. DNA has inherent immunostimulatory properties (see 1.7.2) and therefore may not require formulation with adjuvant. Secondly, the intrinsic stability of DNA means that, depending on the formulation, DNA vaccines can often be stored without refrigeration. One method of administration is particle-mediated DNA delivery (PMDD) using the gene gun (see 1.7.3); this method also obviates the use of hypodermic needles.

Broadly speaking there are two types of genetic vaccines: Viral vectors, including adenoviruses and retroviruses, and naked DNA, including injected plasmid solutions and the gene gun. The former, being least relevant to this study, will be discussed separately and in less detail.

### 1.7.1. Viral vaccine vectors

Recombinant viral vectors are a logical choice as a tool for genetic immunisation because their natural transducing abilities can be exploited. Adenoviral vectors were shown early on to produce significant cellular and humoral responses against encoded antigens(Xiang *et al.*, 1996; He *et al.*, 2000). Long-lasting cellular responses appear to be maintained by the longevity of such vectors within T cells(Tatsis *et al.*, 2007). More recently, adenovirus-based vaccines have shown protection against several different pathogens(Fattori *et al.*, 2006; Lo *et al.*, 2008; Magalhaes *et al.*, 2008; Raviprakash *et al.*, 2008; Resende *et al.*, 2008; Arevalo *et al.*, 2009) as well as tumour antigens(Butterfield *et al.*, 2008; Peruzzi *et al.*, 2009).

A significant disadvantage to viral vector-based vaccines is the presence of viral antigens. Preexisting antibodies to these antigens can neutralise the vector, greatly reducing its efficacy(Yang *et al.*, 1995; Fitzgerald *et al.*, 2003). Similarly, a response generated against an initial administration of vaccine could neutralise later, booster, immunisations. Two approaches to avoid this problem show promise: PEGylation, the modification of viral particles with polyethylene glycol, appears to shield virions from antibodies, allowing them to avoid neutralisation before transfection has occurred(Croyle *et al.*, 2001; Croyle *et al.*, 2002). Secondly, boosting an adenovirally primed response with a vector of a different serotype(Pinto *et al.*, 2003), or an entirely different virus(Bruna-Romero *et al.*, 2001), may circumvent the problem of neutralisation by antibodies generated to the priming vector. However using this route extensively would eventually use up all available viral vectors and so would not be suitable as a general method of immunisation.

Whilst DC remain the most important target for genetic vaccines, adenoviruses do not exhibit a strong affinity to these cells. Several studies have shown improvement of DC targeting by alteration or fusion of viral envelope proteins(Okada *et al.*, 2001; Korokhov *et al.*, 2005) (Pereboev *et al.*, 2004). As well as shielding them from preexisting antibodies, PEGylation also 'detargets' adenoviral vectors from their natural targets: CAR-expressing, non-DC cells(Kreppel *et al.*, 2005; Wortmann *et al.*, 2008). This presumably increases the availability of free vector to be taken up by endocytic DC.

Retroviral vectors have been used to transfect DC *ex vivo*(Chinnasamy *et al.*, 2000; Schroers *et al.*, 2000) and *in vivo*(VandenDriessche *et al.*, 2002; Esslinger *et al.*, 2003; Palmowski *et al.*, 2004). DC transfected *ex vivo* by retroviral vectors have been shown to be effective at stimulating T cell responses(Granelli-Piperno *et al.*, 2000; Dyall *et al.*, 2001; Metharom *et al.*, 2001; Esslinger *et al.*, 2002; He *et al.*, 2005). However, as discussed in 1.6.4, ex vivo treatment of cells followed

by their return to the body is time- and labour-intensive. Administration of viral vectors in vivo is the ideal procedure for this technique and has shown some success[(Esslinger *et al.*, 2003; Palmowski *et al.*, 2004; Rowe *et al.*, 2006; Iglesias *et al.*, 2007; Arce *et al.*, 2009)].

Improvements to first generation lentiviral vectors have increased their potential usefulness as vaccines. The safety profile of such vaccines has been improved by restricting antigen gene expression to DC by placing it under control of the dectin-2 promoter[(Lopes *et al.*, 2008)] and by inserting mutations which prevent integration into the host genome[(Karwacz *et al.*, 2009)]. Safety has also been improved by targeting of vector to APC through fusion of single-chain antibodies to an existing envelope protein[(Gennari *et al.*, 2009)]. Such targeting also improves the immunostimulatory ability of the retroviral vector. Similarly, the encoding of vFLIP, a viral activator of NF-κB, alongside antigen gene induces DC maturation and, therefore, enhances the response to antigen[(Rowe *et al.*, 2009)].

Despite positive results using viral vectors as vaccines they are inherently complex. Pathogenic reactions have arisen in the past[(Raper *et al.*, 2002; Raper *et al.*, 2003)], and the potential remains for recurrences in the future. Naked DNA vaccines involve fewer antigens and the pathways involved are simpler and more well understood. A successful naked DNA vaccine is, therefore, likely to be more attractive than an equally effective viral vector vaccine.

### 1.7.2. DNA as an adjuvant

As mentioned above, naked DNA vaccines do not require formulation with adjuvants such as aluminium hydroxide or IFA. This is primarily due to the presence of unmethylated CpG sequences in bacterial DNA which result in the secretion of both $T_H1$ and $T_H2$ cytokines[(Yamamoto *et al.*, 1992b; Krieg *et al.*, 1995; Pisetsky *et al.*, 1995; Halpern *et al.*, 1996; Klinman *et al.*, 1996)]. The presence of such sequences has been shown to be necessary for the induction of an immune response by DNA vaccines[(Sato *et al.*, 1996; Klinman *et al.*, 1997)]. CpG-rich DNA has, in fact, been used as adjuvant alongside genetic immunisations[(Tengvall *et al.*, 2005)], protein antigen[(Brazolot Millan *et al.*, 1998; Davis *et al.*, 2000; Gallichan *et al.*, 2001; Tengvall *et al.*, 2006)] or even alone prior to viral challenge[(Harandi *et al.*, 2003)]. The importance of the state of methylation of CpG sequences in determining their immunogenicity is unclear[(Klinman *et al.*, 1997; Feltquate and Robinson, 1999; Cornelie *et al.*, 2004b)].

As described in 1.3.1, dendritic cells detect danger signals by the expression of TLR molecules which recognise pathogen-associated molecular patterns. The immunostimulatory effect of CpG sequences is mediated by DC[(Halpern *et al.*, 1996; Jakob *et al.*, 1998; Sparwasser *et al.*, 1998; Behboudi *et al.*, 2000)] and other APC such as B cells[(Messina *et al.*, 1991; Krieg *et al.*, 1995)] following the binding of CpG to TLR-9[(Bauer *et al.*, 2001b; Cornelie *et al.*, 2004a; Lin *et al.*, 2004b; Coch *et al.*, 2009; Ma *et al.*, 2009)]. Although early data showed that bacterial DNA could trigger both $T_H1$ and $T_H2$ cytokine production, more recent studies have shown a clear $T_H1$ bias, most significantly the expression of IL-12 by DC[(Halpern *et al.*, 1996; Jakob *et al.*, 1998; Behboudi *et al.*, 2000; Kranzer *et al.*, 2000; Weeratna *et al.*, 2000; Liu *et al.*, 2005)].

Whilst recognition of bacterial DNA by a Toll-like receptor is unquestionably important, recent work has also identified other, non-TLR receptors which recognise bacterial DNA[(Takaoka *et al.*, 2007; Ishii *et al.*, 2008)]. A caveat to studies of genetic immunisation in murine models is the difference in expression of TLRs between mouse and human cells. In particular, studies have shown that human TLR-9 is expressed on

plasmacytoid, but not monocyte-derived, DC[(Bauer *et al.*, 2001a; Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001; Krug *et al.*, 2001; Hornung *et al.*, 2002)]. However, others argue that the latter do, indeed, express TLR-9[(Hoene *et al.*, 2006)].

### 1.7.3. Naked DNA vaccine vectors

Intramuscular injection of plasmid DNA results in expression of an encoded gene[(Wolff *et al.*, 1990)]. Soon after this observation was made it was shown that encoded antigen can induce an immune response and protect against infection[(Robinson *et al.*, 1993; Ulmer *et al.*, 1993)]. The precise mechanism of DNA uptake, gene expression and antigen presentation is unclear. Whilst there is evidence of T cell activation by directly transfected DC[(Casares *et al.*, 1997; Chattergoon *et al.*, 1998)], it appears that direct transfection is not an absolute requirement and that antigen expressed by other transfected cells is also taken up and presented by DC[(Doe *et al.*, 1996; Fu *et al.*, 1997; Iwasaki *et al.*, 1997b)]. It is likely that, in fact, both pathways are involved.

Immunisation by plasmid injection tends to elicit a $T_H1$-skewed response[(Pertmer *et al.*, 1996; Raz *et al.*, 1996; Feltquate *et al.*, 1997; Toda *et al.*, 2000)], making this approach particularly suitable against intracellular pathogens[(Cornell *et al.*, 1999; Weiss *et al.*, 2000)]. It has also been suggested that administration of allergen by this method could be used to redress the $T_H1$-$T_H2$ balance in allergic disorders[(Hsu *et al.*, 1996; Raz *et al.*, 1996; Toda *et al.*, 2000; Hochreiter *et al.*, 2003; Scheiblhofer *et al.*, 2007)].

Particle-mediated DNA delivery (PMDD) using the gene gun involves the precipitation of vector DNA onto microscopic gold particles. It was first demonstrated as an *in vitro* transformation/transfection technique in plant tissue[(Klein *et al.*, 1987)], algae and yeast[(Sanford, 1988)], cultured mammalian cells[(Zelenin *et al.*, 1989)] and, finally, *in vivo* in mice[(Williams *et al.*, 1991)]. It was soon suggested that the technique be used for genetic immunisation[(Johnston, 1990)] and this was soon realised[(Tang *et al.*, 1992)]. The gene gun was shown to be at least as effective as intramuscular injection, to be more reliable and to consume significantly less DNA[(Fynan *et al.*, 1993; Lai *et al.*, 1995; Weiss *et al.*, 2000; Yoshida *et al.*, 2000)].

The mechanism(s) by which gene gun immunisation results in the activation of T cells has been debated. Studies involving immunisation of parent-into-$F_1$ bone marrow chimeras showed that the key APC in DNA immunisation are haemopoietic in origin[(Corr *et al.*, 1996; Iwasaki *et al.*, 1997b)]. It has been demonstrated by microscopy[(Condon *et al.*, 1996; Larregina *et al.*, 2001)], flow cytometry[(Porgador *et al.*, 1998)], luciferase expression[(Larregina *et al.*, 2001)], RT-PCR[(Larregina *et al.*, 2001)] and antigen-presenting co-culture[(Porgador *et al.*, 1998; Larregina *et al.*, 2001)] that PMDD results in directly transfected DC which migrate to the draining lymph nodes and express and present antigen. Indeed, removal of the skin immediately after PMDD prevents immunisation whilst grafting this skin induces a response in a recipient animal[(Torres *et al.*, 1997; Klinman *et al.*, 1998)]. Finally, transcriptional targeting of DC by placing antigen expression under control of the fascin or dectin-2 promoters confirmed that these directly-transfected DC are the key initiators of the immune response to PMDD immunisation[(Morita *et al.*, 2001; Ross *et al.*, 2003)]. However, it is clear that other skin cells such as keratinocytes are also transfected and that they also play a vital role in the development of a full response to antigen[(Nickoloff and Turka, 1994; Hon *et al.*, 2005; Lauterbach *et al.*, 2006; Vandermeulen *et al.*, 2009)].

It has been known for some time that stressed or necrotic cells can activate dendritic cells and that substances released from these cells can aid in antigen processing[(Gallucci *et al.*, 1999; Shi *et al.*, 2000)]. Among these substances are uric acid[(Shi *et al.*, 2003)] and heat-shock proteins (HSPs)[(Basu *et al.*, 2000)]. The presence of

these, normally intracellular, molecules in the extracellular milleu induces dendritic cell activation and maturation when detected via the Toll-like receptors TLR2 and TLR4[(Vabulas *et al.*, 2002; Flohe *et al.*, 2003; Liu *et al.*, 2003)]. Evidence suggests that DC activated in this manner are $T_H1$-biased[(Flohe *et al.*, 2003; Wan *et al.*, 2003)]. Another HSP receptor, CD91, mediates endocytosis, processing and presentation of HSPs and antigens associated with them via the MHC class I pathway[(Basu *et al.*, 2001)]. More recently a C-type lectin, CLEC9A, was shown to detect necrotic cells and to enhance cross-presentation of their antigens[(Sancho *et al.*, 2009)]. Together, DC maturation and increased asntigen presentation efficiency mean that cellular damage and death have potent adjuvant activity.

Successful transfection by gene gun requires plasmid DNA to be delivered into healthy cells which remain so and are able to express the introduced gene(s). Nevertheless, many surrounding cells are damaged or killed. It is, therefore, likely that cellular damage and death caused during gene gun immunisation would be immunostimulatory. Indeed, the doubling of the DC population in the draining lymph node caused by gene gun administration occurs in the presence or absence of DNA on the beads[(Porgador *et al.*, 1998)]. This, combined with the above described effects of CpG sequences in plasmid DNA (1.7.2), makes the gene gun an effective method of immunisation that provides its own, intrinsic, adjuvant activity.

Whereas injection of DNA vaccines tends to promote a $T_H1$ response, the gene gun has a more $T_H2$ bias[(Fynan *et al.*, 1993; Pertmer *et al.*, 1996; Feltquate *et al.*, 1997; Kwissa *et al.*, 2000; Toda *et al.*, 2000)]. The reason for this difference is not well understood although there is evidence to suggest that the significantly smaller quantity of DNA, and therefore of $T_H1$-inducing CpG sequences, plays a role[(Barry and Johnston, 1997)].

The addition of exogenous CpG oligodeoxynucleotides to gene gun beads carrying antigen-encoding plasmid is able to shift the response to antigen from $T_H2$ to $T_H1$[(Zhou *et al.*, 2003)]. Other work shows that gene gun coadministration of uncoated gold beads alone to the site of an injected DNA-saline vaccine was able to $T_H2$-shift the normally $T_H1$ response[(Weiss *et al.*, 2002)]. Therefore the gene gun is, itself, intrinsically $T_H2$-inducing.

Targeting DC by placing transcription of antigen under the control of the fascin promoter results in a cellular response comparable to that generated via a ubiquitous (eg CMV) promoter[(Ross *et al.*, 2003; Sudowe *et al.*, 2003; Vandermeulen *et al.*, 2009)]. However, the antibody response generated is weaker and/or shows a $T_H1$ bias. This supports the classical model whereby intracellular antigen derived from direct transfection of DC is processed and presented via the MHC class I pathway and exogenous antigen derived from other transfected cells, and subsequently endocytosed by DC, is processed and presented via the MHC class II pathway. Nevertheless, targeting expression to keratinocytes also produces a viable $T_H1$ / cellular response[(Hon *et al.*, 2005; Sudowe *et al.*, 2009; Vandermeulen *et al.*, 2009)]. This suggests that, as well as the above processes, cross presentation allows exogenous antigen to be internalised by DC and presented on MHC class I molecules[(Fu *et al.*, 1997; Albert *et al.*, 1998a; Albert *et al.*, 1998b; Cho *et al.*, 2001)].

It is likely that three routes play a part in PMDD immunisation[(Sudowe *et al.*, 2009)]: 1) DC take up antigen secreted by other cells and present it via the MHC class II pathway. 2) DC take up antigen and cross-

present it via the MHC class I pathway. 3) Directly transfected DC present antigen, that they themselves synthesise, on both MHC class I and MHC class II molecules.

### 1.7.4. Improving genetic immunisations

As described above, merely administering antigen-encoding plasmid is often sufficient to induce an immune response. However, adjustment of, and addition to, genetic vaccines allows this response to be augmented or directed. Such improvements have been made at the genetic level and at that of vaccine delivery.

The coadministration of genes encoding immune active molecules has been widely examined. It has already been noted that genetic vaccines avoid the difficulty and expense of production and purification of recombinant proteins. This advantage is amplified in such antigen-modulator vaccines where an additional, often inherently unstable, protein is required.

Cytokines and chemokines, natural modulators of the immune system, have been used to improve genetic vaccines. Firstly, molecules recognised as being involved in the priming of an immune response appear to play similar roles when coadministered alongside antigen genes. For example, IL-12- or IL-18-encoding plasmid has been demonstrated to improve the CTL response to viral antigen[(Iwasaki *et al.*, 1997a; Kim *et al.*, 1997a) (Lee *et al.*, 2003)]. Indeed, inclusion of IL-12 and IFN-γ genes enhances the $T_H1$ arm of the response whilst IL-4-encoding plasmid promotes a $T_H2$ bias[(Chow *et al.*, 1998)]. Secondly, genes encoding molecules involved in the development and localisation of antigen presenting cells, in particular DC, have been coadministered to enhance the response to antigen through increasing the number and maturity of these cells[(Xiang and Ertl, 1995; Eo *et al.*, 2001; Hu *et al.*, 2004; Nayak *et al.*, 2006; Ma *et al.*, 2007)].

Arguably not a genetic modification, but one involving DNA nonetheless, the coadministration of CpG oligonucleotides on gene gun beads has been shown to improve the immunogencity of such immunisation[(Zhou *et al.*, 2003; Tengvall *et al.*, 2005)].

Rather than actually inducing DC maturation, transfection of DC with the costimulatory molecules CD80/86 artificially enhances their immunostimulatory ability. Coadministration of these genes alongside antigen has been shown to improve the immunogenicity of genetic vaccines[(Corr *et al.*, 1997; Iwasaki *et al.*, 1997a; Kim *et al.*, 1997b)]. Similarly, inducing the upregulation of these molecules by immunising with coadminisered CD154 gene also enhances the response, particularly the cellular response, to antigen[(Mendoza *et al.*, 1997; Ihata *et al.*, 1999)].

Genetic immunisation allows the coadministration of genes encoding intracellular signalling molecules. In DC, NF-κB-inducing kinase (NIK) activates NF-κB, inducing maturation, migration and cytokine production. Coadministration of this gene proved effective in enhancing the response to antigen[(Andreakos *et al.*, 2006)]. Another approach improved the response to genetic immunisation by including the gene for the antiapoptotic Bcl-xL molecule, prolonging the life of transfected DC and, therefore, the duration of antigen presentation[(Kim *et al.*, 2003; Kim *et al.*, 2004)].

The fusion of an antigen gene to a cytokine gene can have similar effects to that of the coadministration of separate genes: Fusion to IL-12 has enhanced the CTL response to model antigen

whilst fusion to IL-18 or IL-4 has shifted the response toward a $T_H1$- or $T_H2$-bias, respectively[(Kim *et al.*, 1997a; Kim *et al.*, 1997c; Maecker *et al.*, 1997; Maecker *et al.*, 2001)].

Fusion genes can also be utilised in more elegant ways. Although DC are transfected by genetic immunisations many other cell types are also transfected. It has been shown that fusion of antigen gene to that of a single chain antibody fragment[(Demangel *et al.*, 2005; Nchinda *et al.*, 2008)] or that of CTLA4[(Boyle *et al.*, 1998; Deliyannis *et al.*, 2000; Nayak *et al.*, 2003)] can target in vivo-synthesised antigen to DC and other APC via DEC-205 or CD80/86 molecules, respectively. This increases the uptake and, therefore, presentation of antigen by APC. On a subcellular level, fusion genes have been used to enhance or modify the processing of antigen. Fusion of antigen gene to that of the invariant chain trimerisation domain forces presentation via the MHC class II pathway, increasing the antibody response to antigen[(Toda *et al.*, 2002; Schneiders *et al.*, 2005; Grujic *et al.*, 2009)]. Similarly, fusion to the LAMP-1 gene causes internalisation, processing and presentation via the MHC class II pathway[(Wu *et al.*, 1995)].

Another modification for enhancing genetic immunisation is the encoding of antigenised antobodies. Insertion of epitope sequences into the complementarity-determining region of IgG allows the display of antigen and stimulation of an immune response without classical presentation on MHC class II molecules[(Gerloni *et al.*, 1997; Gerloni *et al.*, 1999; Schuler *et al.*, 2001)]. This bypasses the problem of haplotype restriction which, especially when using monovalent vaccines, restricts efficacy to subpopulations with certain genotypes.

Control over antigen gene expression is another way in which genetic vaccines are superior to protein-based vaccines. As discussed earlier it is possible to target expression to specific cell types. As discussed, targeting transcription to DC causes a bias to cellular immunity; exploiting this phenomenon might be useful, especially in cases where the inherent $T_H2$-bias of the gene gun is undesirable. One difficulty in using cell-specific promoters is that they often induce lower levels of expression than do more promiscuous promoters such as the CMVie promoter. Coadministration of a transactivator to upregulate expression of antigen gene under the control of a tissue-specific promoter allows long-lasting, tissue specific expression but with high expression normally only seen from promoters such as CMVie[(Li *et al.*, 1999b)].

The second class of improvements made to genetic vaccines is that of delivery. Improved intracellular delivery usually concerns plasmid solution injection rather than gene gun immunisation, primarily because PMDD is, itself, an intracellular delivery method. The containment of plasmid vector within polymer microspheres has been used to improve stability and increase uptake efficiency following injection[(Wang *et al.*, 2004; Nguyen *et al.*, 2008)]. The association of plasmid DNA with high molecular weight cationic polymers improves its immunogenicity; this is, at least in part, due to increased uptake by DC[(Chamarthy *et al.*, 2003; Mollenkopf *et al.*, 2004; O'Hagan *et al.*, 2004; Jia *et al.*, 2008)]. Finally, the permeablisation of cells using femtosecond laser pulses has been shown to increase the response following injection of plasmid vector solution[(Zeira *et al.*, 2007)].

## 1.8. Rationale, hypothesis and introduction to the PhD project

The rationale for the main part of this project is derived from three observations: 1) That regulatory T cells, specifically type 1 ($T_R1$) regulatory T cells, can be induced by activation in the presence of IL-10. 2) That dendritic cells secreting IL-10, either naturally or artificially, can induce the induction of regulatory T cells. 3) That a major mechanism of PMDD-mediated immunisation is the direct transfection of skin dendritic cells. The hypothesis under invesigation is that coadministration of antigen- and IL-10-encoding genes in a PMDD immunisation is able to produce IL-10-secreting dendritic cells which present antigen to naïve T cells in the presence of IL-10, thereby tolerising them.

This part of the project aims to characterise gene gun (PMDD) immunisation in a murine, TCR-transgenic, adoptive transfer model and to observe the effect of the inclusion of the interleukin-10 gene in this immunisation. Similar models have been used previously and provide a system in which antigen-specific and –nonspecific T cell responses may be observed and compared, whilst not overwhelming lymphoid tissues with an entirely transgenic T cell population(Creusot *et al.*, 2001; Creusot *et al.*, 2003a; Creusot *et al.*, 2003b). The early T cell response in draining lymph nodes will be examined initially. The long-term effects of coadministration of the IL-10 gene will then be studied. Specifically, the question will be asked whether treatment with the IL-10 gene during a primary immunisation has an impact on later antigenic challenge. The magnitude and kinetics of the T cell proliferative and cytokine responses will comprise the core data collected.

The hypothesis of the second part of this project is that the same PMDD immunisation, either via induction of $T_R1$ cells or another mechanism, is able to protect mice from the induction of allergic airway hyperresponsiveness.

The study will utilise an existing murine model of antigen-specific allergic airway inflammation. The induction of airway inflammation involves priming the animal with antigen prior to an airway challenge with the same antigen. The goal is to understand whether earlier immunisation with antigen affects the outcome of inflammation induction and whether the coadministration of IL-10 modulates this outcome. As well as T cell proliferative and cytokine responses, antibody isotype levels and parameters specific to the airway inflammation model, such as cellular infiltration to the lung, will be examined.

# 2. Materials and Methods

## 2.1. Plasmids

### 2.1.1. The p7 vector

The p7 family of vectors contain a CMV immediate early-based promoter and transfectants are selectable due to the presence of the Tn903 kanamycin resistance gene (Figure 2-1). The gene encoding chicken egg albumin (OVA), the gene encoding murine interleukin-10 (IL-10) and the gene encoding human interferon gamma (hIFN-γ) were independently cloned into the multiple cloning site (MCS) of this vector. The resulting plasmids are referred to as p7.OVA, p7.IL10 and p7.hIFNg, respectively.

### 2.1.2. Synthesis of the OVA gene with 'sticky ends'

The sequence of the chicken egg albumin gene (*ova*) cDNA (Entrez Nucleotide Accession N$^{o}$ V00383.1) is shown in Figure 2-4. The pVAC.OVA vector containing this gene was kindly provided by Ian Catchpole, GlaxoSmithKline, Stevenage, UK. To obtain the *ova* fragment the ova.1 and ova.2 primers (Table 2-1) were used in a standard PCR reaction.

The PCR mixture (50 µl total) contained 5 µl buffer (final conc 1x; Promega), 5 µl dNTP mixture (dATP, dCTP, dGTP, dTTP each at 2 mM final conc; Promega), 2 µl of each primer (final conc 5 µM), 1 µl Pfu DNA polymerase (Promega M7741). An aliquot of the product was analysed by agarose gel electrophoresis (Figure 2-2a) which showed the amplified fragment to be of the expected size of 1100-1200 bp. The remaining product was run on an agarose gel, the band cut out and purified using the QIAquick gel extraction kit (Qiagen 28704) according to the manufacturer's guidelines.

The purified product was digested in the a reaction mixture (total 50 µl) containing 30 µl purified product, 3 µl Not1, 3 µl BamH1, 5 µl 10x BamH1 buffer, 1 µl BSA (all from New England Biolabs). The mixture was incubated at 37°C for 3 h. The product was purified by agarose gel electrophoresis as above.

### 2.1.3. Synthesis of the IL-10 gene with 'sticky ends'

The sequence of the murine interleukin-10 gene cDNA (Entrez Nucleotide Accession N$^{o}$ M37897.1) is shown in Figure 2-5. The Pcmv&muIL10 vector containing this gene was kindly provided by Vincent Hart, GlaxoSmithKline, Stevenage, UK. The *il10* gene had previously been cloned into this vector using Not1 and BamH1 and thus could be obtained by restriction disgest. The digestion mixture (50 µl total) contained the same as that above but with 10 µl vector (final conc 200 ng/µl). The total product was run on an agarose gel to separate *il10* from the backbone vector and the relevant fragment was cut out and purified as above. The result was verified by agarose gel electrophoresis (Figure 2-2b).

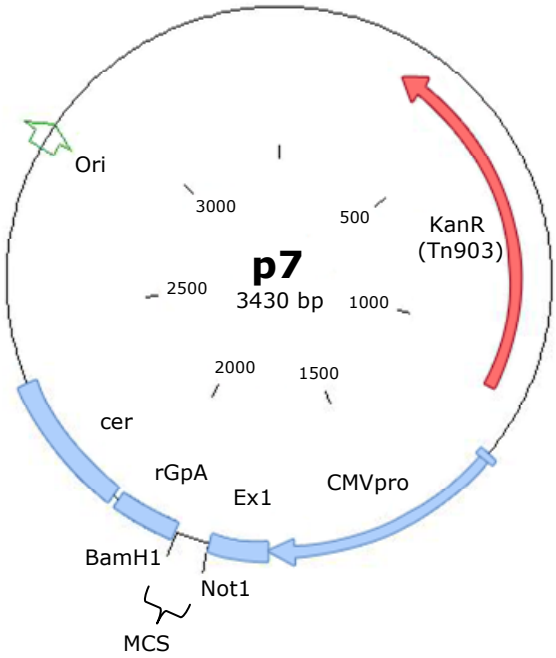



**Figure 2-1. The p7 expression vector.**
The key features of the vector for this study: MCS = Multiple Cloning Site; contains several restriction sites delimited by BamH1 and Not1 restriction sites. CMVpro = cytomegalovirus immediate early promoter; upstream of the MCS; controls expression of inserted gene(s). KanR = Kanamycin resistance gene; allows selection of successfully transfected clones.

| Primer | Direction | Sequence | Restriction site |
|---|---|---|---|
| Cloning of p7.OVA | | | |
| ova.1 | forward | ATAAGAATGCGGCCGCCATGGGCTCCATC | Not1 |
| ova.2 | reverse | CGGGATCCTTAAGGGGAAACACATC | BamH1 |
| Cloning of p7.IL10 | | | |
| IL10.1 | forward | ATAAGAATGCGGCCGCCATGCCTGGCTCAGCACTGC | Not1 |
| IL10.2 | reverse | CGGGATCCTTAGCTTTTCATTTTGATCATCATGTATGC | BamH1 |
| Cloning of p7.hIFNg | | | |
| hIFNg.1 | forward | ATAAGAATGCGGCCGCATGAAATATACAAGTTAT | Not1 |
| hIFNg.2 | reverse | CGGGATCCTTACTGGGATGCTCTTC | BamH1 |

**Table 2-1. Primers used in the cloning of genes into the p7 vector.**
Underlined bases comprise the restriction site named in the last column. All primers were obtained from MWG Biotech AG, Ebersberg, Germany.

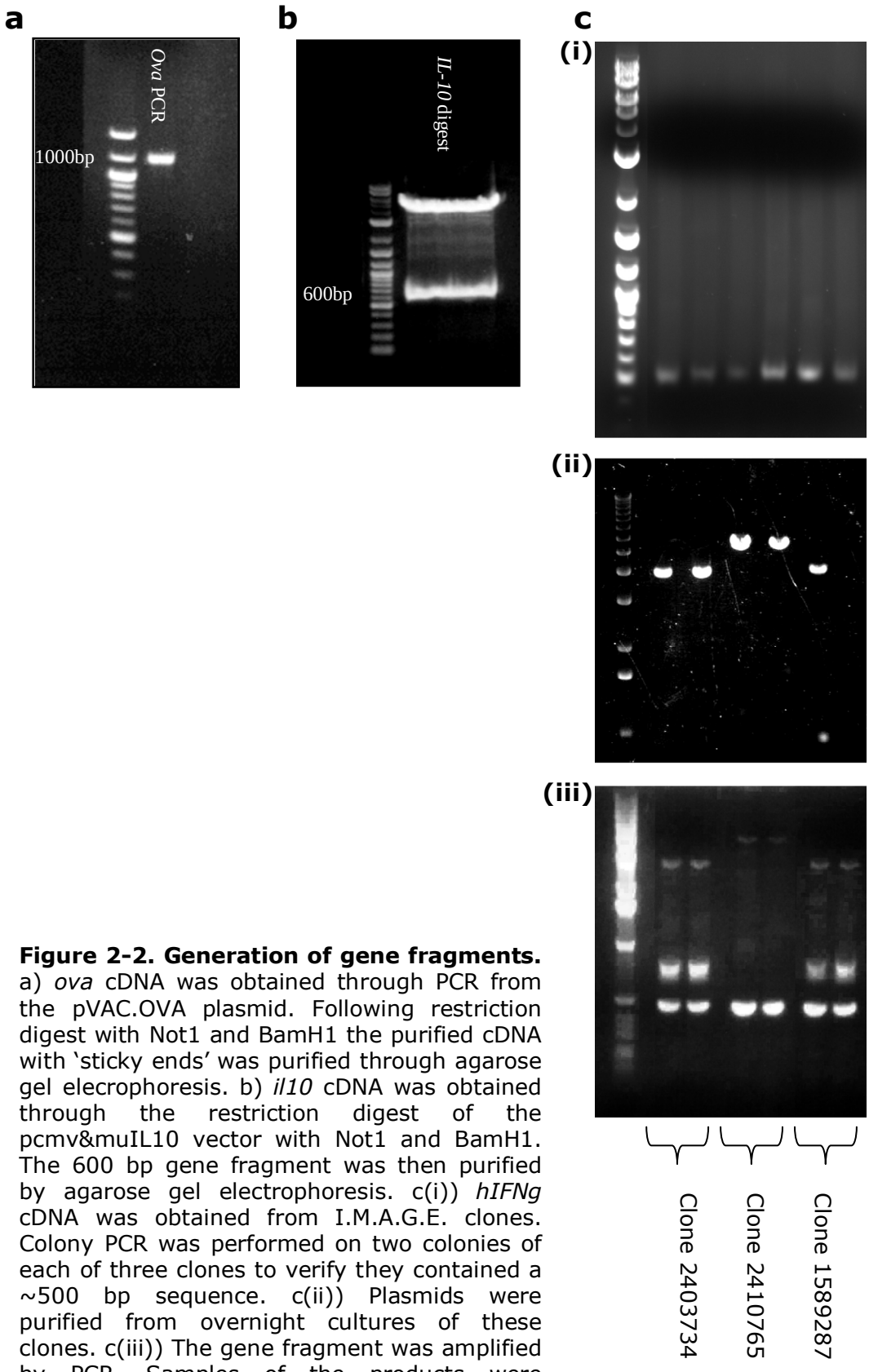



**Figure 2-2. Generation of gene fragments.** a) *ova* cDNA was obtained through PCR from the pVAC.OVA plasmid. Following restriction digest with Not1 and BamH1 the purified cDNA with 'sticky ends' was purified through agarose gel elecrophoresis. b) *il10* cDNA was obtained through the restriction digest of the pcmv&muIL10 vector with Not1 and BamH1. The 600 bp gene fragment was then purified by agarose gel electrophoresis. c(i)) *hIFNg* cDNA was obtained from I.M.A.G.E. clones. Colony PCR was performed on two colonies of each of three clones to verify they contained a ~500 bp sequence. c(ii)) Plasmids were purified from overnight cultures of these clones. c(iii)) The gene fragment was amplified by PCR. Samples of the products were analysed by agarose gel electrophoresis. The fragment from clone 2410765 was digested with Not1 and BamH1 and repurified.

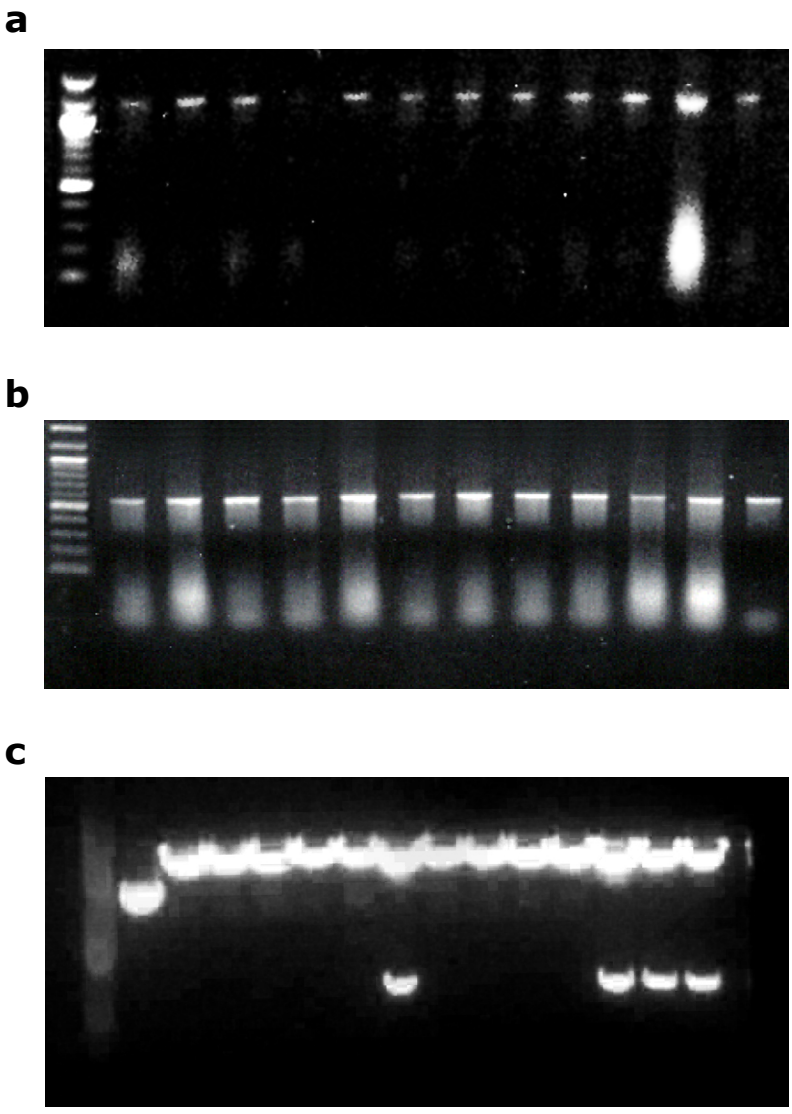



**Figure 2-3. Confirmation of insertion of fragments by colony PCR (cPCR) or restriction digest.**
cPCR was performed by inoculating cells from an individual colony into the PCR mixture and then into a separate culture. PCR products were run on agarose gel to identify successful insertion of p7.OVA (a) or p7.IL10 (b). A corresponding culture was subsequently grown up for plasmid preparation. cPCR was not successful on p7.hIFNg clones so minipreps were prepared from each clone and PCR performed on each preparation. These were then run on agarose gel to identify sucessful clones by the presence of the inserted fragment (c). A successful clone was subsequently grown up for plasmid preparation.


**Figure 2-4. Sequence of cloned ovalbumin gene compared to that published in the NCBI GenBank.**
The GenBank sequence (V00383.1) is displayed with cloned gene mutant codons inserted between lines. Substituted bases are highlighted in red. Primers used to clone the gene are displayed in blue. The coding sequence is 1161 bp.


**Figure 2-5. Sequence of cloned IL-10 gene compared to that published in the NCBI GenBank.**
The GenBank sequence (M37897.1) is displayed with cloned gene mutant codons inserted between lines. Substituted bases are highlighted in red. Primers used to clone the gene are displayed in blue. The coding sequence is 537 bp.

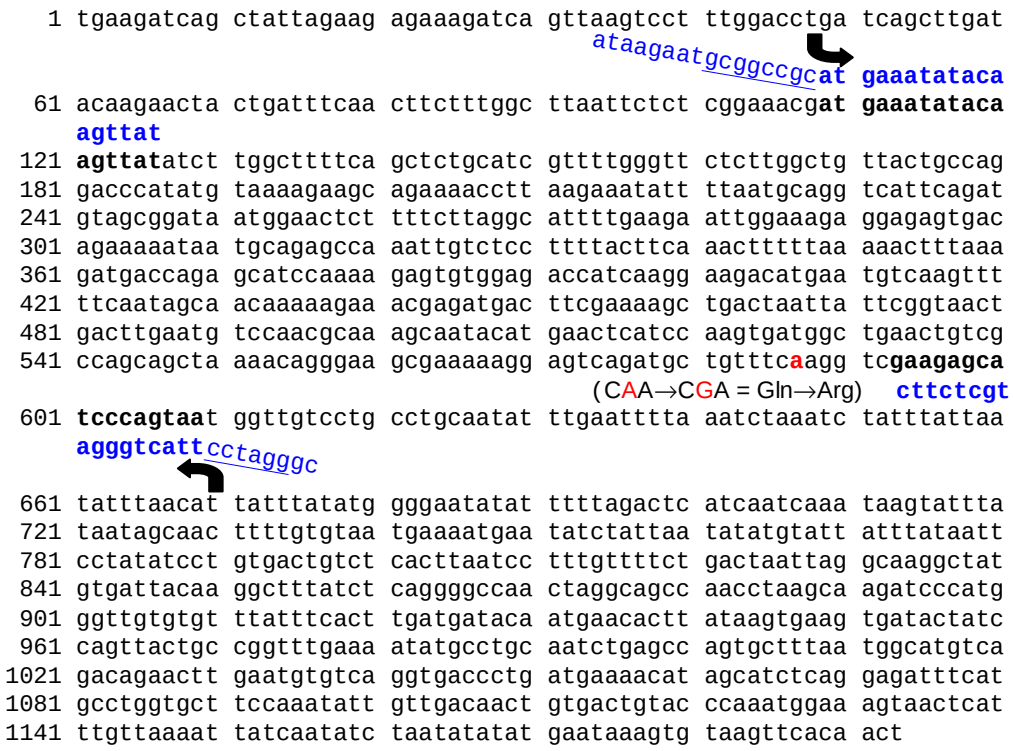



**Figure 2-6. Sequence of cloned interferon-γ gene compared to that published in the NCBI GenBank.**
The GenBank sequence (X13247.1) is displayed with cloned gene mutant codons inserted between lines. Substituted bases are highlighted in red. Primers used to clone the gene are displayed in blue. The coding sequence is 501 bp.

### 2.1.4. Synthesis of the human IFN-γ gene with 'sticky ends'

The sequence of the human interferon-γ gene cDNA (Entrez Nucleotide Accession N$^{o}$ X13247.1) is shown in Figure 2-6. The gene sequence was BLAST searched against the expressed sequence tagged database and three potential clones were identified in the I.M.A.G.E. database (Table 2-2). They were obtained from the HGMP resource centre.

Each clone was streaked onto Luria Bertani (LB) agar containing 100 μg/ml ampicillin and allowed to grow overnight at 37°C. Two colonies of each clone were picked and split into a colony PCR and an overnight culture as follows: Each colony PCR mixture (total 10 μl) contained 1 μl 10x buffer (Promega), 1 μl dNTP mixture (dATP, dCTP, dGTP, dTTP each at 2 mM final conc; Promega), 1 μl of each primer (final conc 5 μM) and 1 μl Pfu DNA polymerase (Promega M7741). The hIFNg.1 and hIFNg.2 primers used are described in Table 2-1. The picked colony was dipped into this mixture and vigorously mized, which provided sufficient copies of the clone for the reaction. PCR products underwent agarose gel electrophoresis which confirmed the presence of the ~500 bp cDNA (Figure 2-2c(i)).

The remainder of the colony was dropped into 4 ml LB medium containing 100 μg/ml ampicillin and allowed to grow for 19 h at 37°C with shaking. Plasmids were purified from the cultures using the QIAprep spin miniprep kit (Qiagen 27104) and repurified via agarose gel electrophoresis (Figure 2-2c(ii)). All three clones contained plasmids. 2403734 and 1589287 which are described by HGMP as containing the same plasmid contained plasmids of the same size whereas 2410765 which is described as containing a different plasmid contained a larger plasmid.

On each purified plasmid was performed two separate PCRs using the primers hIFNg.1 and hIFNg.2 under the conditions described for the *ova* fragment and an aliquot of each product was analysed by agarose gel electrophoresis (Figure 2-2c(iii)). The remainder of the PCR product from clone 2410765 was purified by gel electrophoresis, digested and repurified as described above for the *ova* fragment.

### 2.1.5. Ligation of fragments into p7

The p7 vector was kindly supplied by Peter Ertl, GlaxoSmithKline, Stevenage, UK. Each insert had been cloned or digested to have Not1 and BamH1 restriction sites as described in 2.1.2, 2.1.3 and 02.1.4. The 'open', 'empty' vector with sticky ends was obtained by removal of this insert by restriction digest and agarose gel purification as described above. Each gene was independently ligated into the empty vector following the setup in Table 2-3, whereby five reactions provide an internally-controlled ligation.

Ligation mixtures (total 10 μl) contained 1 μl T4 buffer, 1 μl open p7, 1 μl T4 ligase (Promega M180A; except negative control) and 0, 0.5, 1 or 2 μl of gene fragment. Ligation was allowed to proceed at 4°C overnight.

| Entrez Nucleotide Accession N$^{o}$ | IMAGE clone |
|---|---|
| AI862844 | 2410765 |
| AI769523 | 2403734 |
| AA946992 | 1589287 |

**Table 2-2. Clones of human IFN-γ gene acquired.**
Clones were matched by searching the BLAST database. Three I.M.A.G.E. clones were chosen. Clones were acquired from the HGMP resource centre.

| | Cut p7 vector | Insert | T4 ligase | Buffer | $H_20$ | Total | |
|---|---|---|---|---|---|---|---|
| **1** | 1 µl | - | - | 1 µl | 8 µl | 10 µl | Control against uncut p7 vector |
| **2** | 1 µl | - | 1 µl | 1 µl | 7 µl | 10 µl | Control against self-ligation of p7 vector |
| **3** | 1 µl | 0.5 µl | 1 µl | 1 µl | 6.5 µl | 10 µl | ↓ Increasing quantity of insert to demonstrate that successful ligation (colony count) is dependent on insert |
| **4** | 1 µl | 1 µl | 1 µl | 1 µl | 6 µl | 10 µl | |
| **5** | 1 µl | 2 µl | 1 µl | 1 µl | 5 µl | 10 µl | |

**Table 2-3. Outline of ligation reactions.**
Successful ligation of each insert into the p7 vector was determined by several controls. Reaction 1 controls for presence of uncut p7 vector. Reaction 2 controls for p7 vector which has religated with itself; either due to failure of one restriction enzyme or contamination with the originally removed MCS fragment. Reactions 3-5 contain increasing amounts of insert to determine the optimum amount of insert for ligation efficiency.

### 2.1.6. Transfection into competent host cells and production of plasmids

30 µl XL1-Blue chemically competent E. coli (Stratagene, 200268) were allowed to thaw on ice for 1 h. 1 µl ligation product was added to the cells and the mixture was left on ice for another 1 h. The cells were heat-shocked by incubating in a waterbath at 42°C for 30 s and immediately returning them to ice for 2 mins. They were then allowed to recover in 1 ml Super Optimal Broth with Catabolite Repression (SOC) medium at 37°C for 1 h before plating on LB agar plates containing 50 µg/ml kanamycin and incubating overnight at 37°C. Ligation/transfection was deemed successful if the number of resulting colonies was dependent on the quantity of insert in the ligation reaction. In each case, there were few or no colonies from ligation 1 or 2, and increasing numbers of colonies from ligations 3-5.

To verify further the cloning, several colonies were picked, some cells transferred to a marked spot on a LB agar plate containing 50 µg/ml kanamycin and the remainder dropped into a colony PCR (as above). p7.OVA colony PCR used the ova.1 and ova.2 primers, p7.IL10 the IL10.1 and IL10.2 primers and p7.hIFNg the hIFNg.1 and hIFNg.2 primers (see Table 2-1). Colony PCR of p7.hIFNg clones was unsuccessful so PCR was performed on purified DNA, isolated from the clones using using the QIAprep spin miniprep kit (Qiagen 27104).

PCR products were analysed by agarose gel electrophoresis. Colonies that contained the cloned gene were distinguishable by a bright band of the correct molecular weight. There were 11/12 p7.OVA positives at around 1100 bp, 12/12 p7.IL10 positives at around 500 bp and 4/13 p7.hIFNg positives at around 500 bp (Figure 2-3). One insert-positive colony for each plasmid was selected, grown in LB medium containing 50 µg/ml kanamycin and plasmid purified for sequencing using the QIAprep spin miniprep kit (Qiagen 27104).

Inserts in each plasmid were sequenced at the internal GSK sequencing facility. Sequences were compared with the original BLAST sequences. The *ova* gene contained two silent mutations and one substitution (threonine to alanine) mutation (Figure 2-4). The *il10* gene contained a silent mutation (Figure 2-5). The *hIFNg* gene contained one glutamine to arginine substitution (Figure 2-6). The sequences were deemed accurate enough to proceed with the study and a functional quantity of endotoxin-free plasmid was prepared using the EndoFree plasmid giga kit (Qiagen 12391), following the manufacturer's instructions.

## 2.2. Cartridges for PMDD

### 2.2.1. Plasmid ratios

The dose per mouse of p7.OVA and p7.IL10 to be used are established as described in the subsequent chapters. To ensure consistent DNA-loading efficiency and to control for any immunotimulatory effects of plasmid DNA itself, all cartridges used contained a total of 1 µg DNA on 0.5 mg gold beads. Where the total quantity of gene-expressing plasmid required was less than 1 µg per cartridge, the difference was made up with empty p7 vector.

### 2.2.2. Cartridge preparation

Gold particles of mean diameter 2μm (PowderJect) were sonicated in the presence of 0.05M spermidine (Sigma S0266) for 30 s. The volume of spermidine used was 100μl if the total volume of DNA to be added was less than 100 μl. If the required DNA volume was greater than 100 μl, an equal volume of spermidine was used. Plasmids were added in appropriate combinations so that a total DNA loading rate (DLR) was always 2 μg DNA per mg gold (Plasmid (μg) = Gold (mg) x DLR). To allow multiple plasmids to be loaded onto each bead, the total DLR value (2) is divided appropriately, the calculation is performed on the sub-DLR for each plasmid and the amount of each plasmid to be added is obtained. This was followed by addition of a volume equal to that of the spermidine of 1M $CaCl_2$ with immediate thorough vortexing and a 10 min rest, ensuring efficient precipitation of DNA onto the gold particles. The DNA-coated gold particles were washed three times in dehydrated absolute ethanol and resuspended in 0.05M polyvinylpyrrolidone (PVP, Sigma) in dehydrated absolute ethanol to 8.75 mg gold/ml. The suspension was injected into and adsorbed onto the inner surface of Tefzel tubing (TFX Medical Inc, NH, USA) by centrifugal force using a tube turner device (PowderJect, Chiron Corporation, Madison, WI, USA). The tubing was left to dry and cut into 1.27 cm cartridges which were stored desiccated at 4°C.

### 2.2.3. Cartridge preparation for linkage experiments

For the linkage experiments that examined the requirement for plasmids to be coated on the same bead, a more complex arrangement was required to ensure effective controls. Four, rather than two, cartridges were administered per mouse (see 2.7.3). Using the arrangement in Figure 2-7 it was possible to immunise with p7.OVA+p7.IL10 on the same bead, on separate beads in the same cartridge and in separate cartridges whilst keeping the quantity of both plasmids, as well as total DNA, constant.

### 2.2.4. Quality control

Two cartridges from each batch were tested for loading by eluting DNA in 50μl water/cartridge and measuring its concentration by spectrophotometry. Cartridges routinely contained 0.5-0.75 μg DNA.

## 2.3. Cell culture

### 2.3.1. MC57 murine fibrosarcoma

MC57, a murine fibrosarcoma cell line, was kindly provided by P. Gilboy, GlaxoSmithKline. It was propagated in complete (excluding 2-mercaptoethanol) Dulbecco's Modified Eagle Medium (Invitrogen-Gibco 11960044) with regular passage via trypsinisation (0.025% trypsin solution, 2 min), washing and replating.

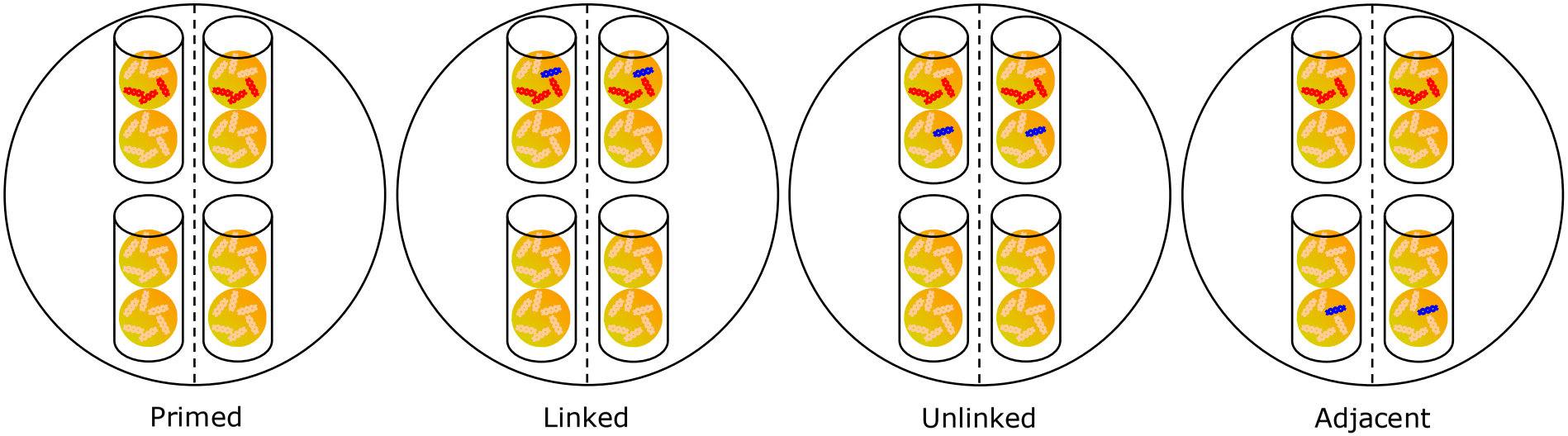



**Figure 2-7. Plasmid distribution for properly controlled linkage experiments.**
In order to properly control the linkage experiments, it was necessary to administer 4, rather than 2, PMDD cartridges per mouse. Each circle represents the abdomen of a mouse; the dotted line being the invisible mid-point.
The "primed" group received 0.5 µg p7.OVA. The "linked" group received 0.5 µg p7.OVA + 0.17 µg p7.IL10 on the same bead. The "unlinked" group received 0.5 µg p7.OVA + 0.17 µg p7.IL10 on separate beads in the same cartridge. The "adjacent" group received 0.5 µg p7.OVA + 0.17 µg p7.IL10 in separate cartridges.
The arrangement used allowed the quantity of p7.OVA and of total DNA per mouse to be constant throughout all groups, and for the quantity of p7.IL10 per mouse to be constant throughout all groups that received that vector. The quantity of each plasmid per bead and the number of beads carrying each plasmid is also constant, controlling both the number of p7.OVA- and p7.IL10-transfected cells and the number of each of these vectors that each cell receives.

### 2.3.2. Preparation of murine bone-marrow derived dendritic cells

Day 1: Femurs and tibeas were collected from both legs, sprayed with ethanol and temporarily stored in complete Modified Eagles Medium. Distal sections of bones were removed and, using a syringe of medium with needle, the lumen of the bone was punctured and the marrow scraped/forced out into a clean dish. The mixture was pipetted up and down to break up any clusters of cells and transferred to a 50 ml centrifuge tube. The mixture was left for 3 min to allow clumps to fall out of suspension before the suspension was transferred to a new tube. Cells were centrifuged and resuspended in red blood cell-lysing buffer for 5 min before being washed, counted and resuspended to 5-7x$10^5$ cells/ml in complete Iscove's medium (Invitrogen) containing 25mM Hepes and 20ng/ml GM-CSF (PeproTech). 3 ml cell suspension was added to wells of a 6-well plate (Nunc 140675) and incubated for 72 h.

Day 4: Supernatant (containing the non-adherent population such as B cells) was gently swirled and carefully removed. 3 ml fresh medium (including fresh GM-CSF) was added to each well.

Day 7/8: Dendritic cells, having now detatched from the well floor, were obtained by gentle pipetting up and down. They were then ready for use after washing and counting.

### 2.3.3. *In vitro* expansion of DO11.10 cells

One DO11.10 spleen was harvested and a cell suspension prepared by homogenisation using a 70 μm cell-strainer (Falcon 2350). This was cultured in complete RPMI plus 250 nM $OVA_{323-339}$ at 37°C and 5% $CO_2$. On day 2, cells were split 1:1 into fresh medium and recombinant IL-2 (PeproTech EC 212-12) was added to give a final concentration of 10 ng/ml. On day 4, cells were split 1:4 and fresh IL-2 was added to the same concentration. Cells were collected for use on day 7.

## 2.4. *In vitro* transfection

### 2.4.1. Transfection of Chinese Hamster Ovary (CHO) cells

CHO cells were grown in complete Dulbecco's Modified Eagle Medium GlutaMAX (Invitrogen-Gibco 10566-016). The day before transfection, cells were seeded at $10^5$ cells per well of a 24-well plate. Transfast reagent (Promega E2431) was prepared and cells transfected according to manufacturer's guidelines. Briefly, medium was removed from the cells before addition of a thoroughly vortexed mixture of 1 μg DNA, 6 μl Transfast reagent and 200 μl medium per well. Transfection was allowed to proceed for 1 h at 37°C before cells were overlaid with 1.5ml medium and incubated for a further 48 h. Supernatants were harvested and stored at –20°C.

### 2.4.2. *In vitro* gene gun of the MC57 murine fibrosarcoma cell line

Before PMDD MC57 cells were reconstituted to 200x$10^6$ cells/ml. 20 μl (4x$10^6$ cells) was placed in a small droplet in a well of a 6 well plate (Falcon 3046) and spread to a diameter of 1 cm. The end of the barrel was positioned 1 cm above the droplet. One cartridge was fired at the cells using compressed

helium at 275 psi. Cells were allowed to recover for 6 min and were then washed in 1 ml medium. Cells were transferred to wells of a 24-well plate (Nunc 143982) in 2 ml medium.

### 2.4.3. *In vitro* gene gun of bone-marrow derived dendritic cells

Polylysine-coated microscopy coverslips were prepared under sterile conditions: For each transfection, a microscopy coverslip was sterilised with ethanol and allowed to dry. Coverslips were coated in polylysine by dipping in a 0.1% (w/v) solution of poly-L-lysine hydrobromide (MW >300,000; Sigma, P-1524) for several seconds. Excess polylysine was removed by dipping several times in sterile distilled water. The corner of the coverslip was dabbed on sterile tissue and the coverslip placed on its side against the wall of a well of a 6-well plate. Despite prior sterilisation of the coverslips, the polylysine is not sterile and cannot be autoclaved or filter-sterilised. Thus, once dry, the open plate was UV-irradiated ensuring that both sides of each coverslip were exposed for at least 15 min. Coverslips were used within 24 hours of preparation although they are believed to be stable for over a week.

For *in vitro* use, the standard gene gun barrel was modified to diffuse the power of the shot across the target site (Figure 2-8). The thread from a 50 ml centrifuge tube was attached to the outside of the end of the gene gun barrel liner. The central section of the lid of the centrifuge tube was cut out, leaving a screw-on cap which would allow the DNA-loaded gold particles to pass through. Between the barrel liner and this cap was sandwiched the diffuser, obtained by cutting out the base of a 70 μm cell strainer (Falcon 2350). During the design process, the effectiveness of the modification was established by firing the gene gun into a sheet of Nescofilm (Karlan Research Products Corporation, USA) (Figure 2-9). Once diffusion had been observed in this way, the apparatus was tested on cells as described below:

Bone marrow-derived dendritic cells were prepared as in 2.3.1. On day 7, DC were washed, counted and resuspended to $5x10^6$ cells/ml. Polylysine-coated coverslips were placed flat at the bottom of wells of a 6-well plate and 200 μl DC suspension ($1x10^6$ cells) was carefully placed at the centre of each coverslip. The droplet was normally 1 cm in diameter. The plate was closed and incubated for 2 hours in saturated humidity to allow DC to adhere whilst preventing evaporation.

Just before transfection, the droplet of medium was drained, leaving the DC attached to the polylysine-coated coverslip exposed. The end of the modified barrel was positioned around the patch of cells, touching the bottom of the well. The diffuser was therefore suspended 2-4 mm above the cells. One cartridge was fired at the cells at 500 psi. The diffuser was replaced and the barrel sprayed with methylated spirits between shots to prevent contamination. One minute after PMDD the cells were gently covered with 2 ml complete Iscove's medium containing 25mM Hepes and 2ng/ml GM-CSF medium and incubated for 24 or 48 h.

Extensive testing showed the modified gene gun barrel was more effective for *in vitro* PMDD than merely reducing the helium pressure; reducing pressure decreased the diameter of the shot site whereas the modified barrel produces an equal sized shot site with more homogenous gold density throughout.

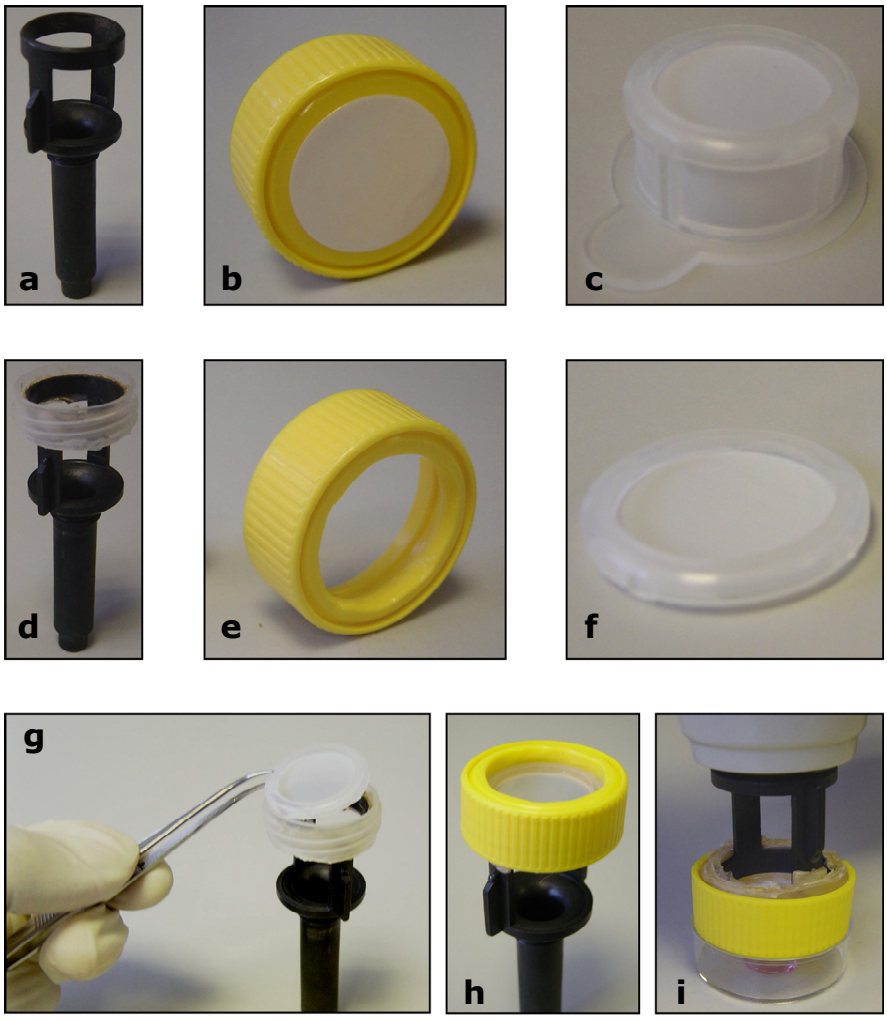



**Figure 2-8. Modification of the gene gun barrel for in vitro PMDD.**
(a) The original barrel of the gene gun. (b) The lid of a 50ml centrifuge tube. (c) A 70μm cell strainer. (d) The threaded top of a 50ml centrifuge tube was removed and glued to the end of a gene gun barrel. (e) The central section of the centrifuge tube lid was removed. (f) The base of a 70μm cell strainer was removed to produce a blast diffuser. (g) The diffuser was placed on the end of the gene gun barrel and (h) sealed in place by screwing on the modified lid. (i) The modified gene gun barrel was installed onto the loaded gene gun and used to transfect 4x106 MC57 cells in a droplet of 20 μl medium.

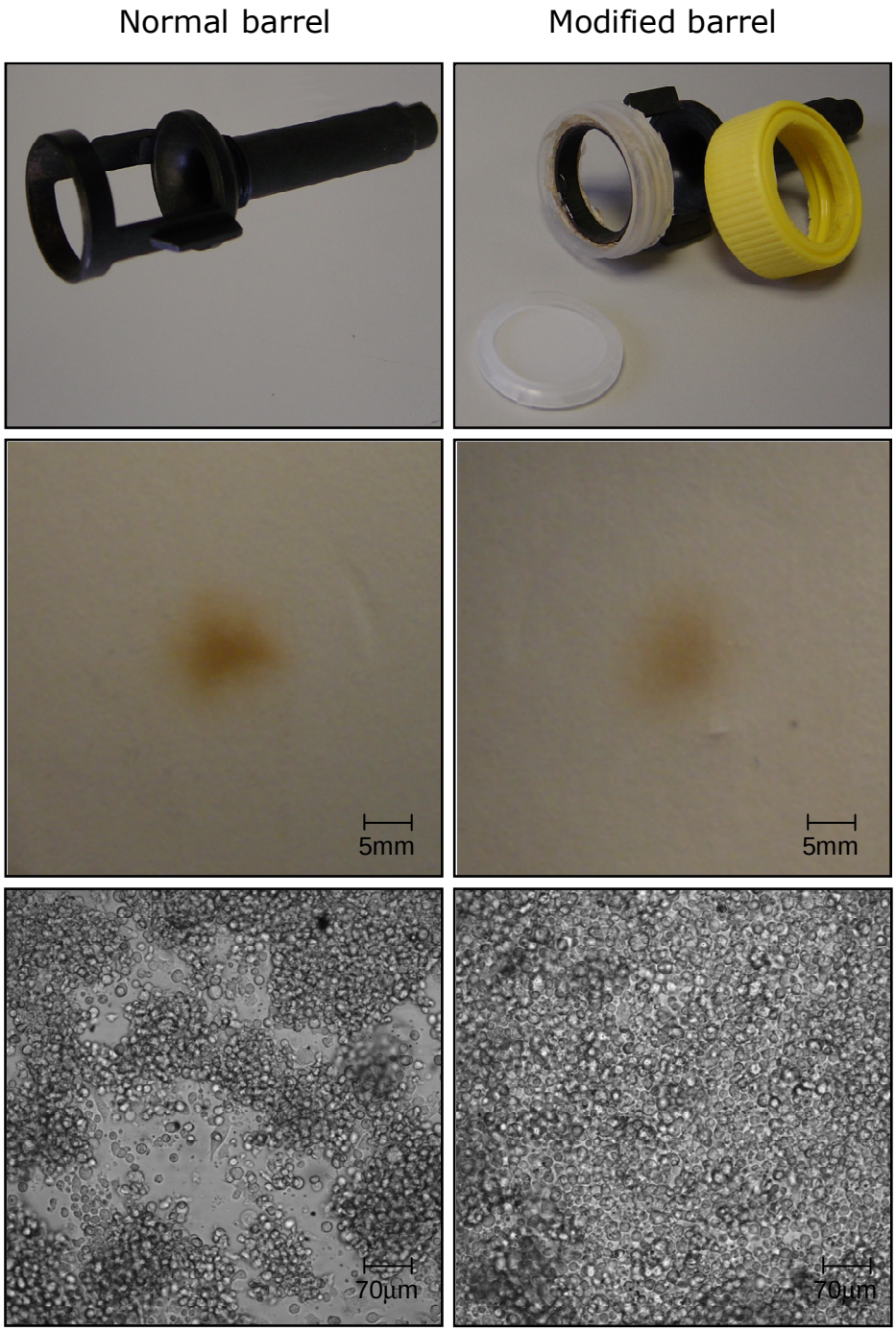



**Figure 2-9. Testing of the gene gun barrel modified for *in vitro* PMDD.** The gene gun barrel and its modified equivalent (top). The ballistics of the modified and unmodified barrel were examined by firing a cartridge into a sheet of Nescofilm (middle). Microscopic examination of transfected cells after recovery showed the extent of cell damage caused by each apparatus (bottom).

## 2.5. Confocal fluorescence microscopy

### 2.5.1. Confocal fluorescence microscopy of bone marrow-derived DC

24 h following *in vitro* PMDD, the coverslip onto which the DC had adhered during incubation was removed from its tissue culture well and washed by dipping in PBS. DC were simultaneously nucleus-stained and mounted by simply placing the coverslip cell-side-down onto a droplet of mounting medium on a microscope slide. Mounting medium was Vectashield containing 1.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories | H-1200). Cells were visualised by confocal microscopy on a Leica SP2 AOBS (acoustica optical beam splitter) system.

## 2.6. Western Blot

Samples were run, alongside Rainbow molecular weight markers (Amersham Life Science RPN-800) on a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham Biosciences RPN2020D). The membrane was blocked in 5% milk powder in PBST. It was then incubated with a 1:500 dilution of polyclonal rabbit anti-OVA (generated by Prof B. Chain) in PBST/2% milk for one hour at room temperature with shaking. The membrane was washed thoroughly with PBST and incubated with a 1:3000 dilution of swine anti-rabbit horseradish peroxidase conjugated polyclonal antibody (DAKO P0399) in PBST/2% milk for one hour at room temperature with shaking. Further washes were performed before the blot was visualised by rinsing with ECL reagent (Amersham Biosciences RPN2109) and exposing to photographic film (Amersham Biosciences RPN3103K). Gels were stained with Coomassie Brilliant Blue to confirm sample loading consistency.

## 2.7. *In vivo* work

### 2.7.1. Mice

DO11.10 mice (Balb/c background, H-2d haplotype), donors of the ovalbumin ($OVA_{323-339}$) epitope-specific transgenic T cells[(Murphy *et al.*, 1990)], were originally obtained from Prof K. Murphy (Washington University School of Medicine, St Louis, MO, USA) and a colony was maintained at The Frythe, GlaxoSmithKline, Welwyn, UK. Balb/c mice were obtained from Charles River UK or Harlan UK. All experiments were carried out under UK ethical guidelines. Unless stated otherwise, mice were sacrificed by cervical dislocation.

### 2.7.2. Adoptive transfer of DO11.10 cells

For every three Balb/c recipients, one DO11.10 spleen was harvested and a single-cell suspension prepared by homogenisation using a 70μm cell-strainer (Falcon 2350). Cells were washed once in HBSS (Invitrogen-Gibco 14175-053) and resuspended in 1 ml red blood cell lysis buffer (Sigma R7757) per spleen. Gentle agitation was applied for 2 min at which time a large volume of HBSS was added to arrest lysis. The cells were pelleted, resuspended in 40ml HBSS and counted using trypan

blue (Sigma T8154) and a haemocytometer. $5x10^5$ cells were stained with anti-mouse CD4-TC and the clonotypic antibody KJ1.26-PE. These cells were analysed on a flow cytometer (FACScan™, Becton Dickenson) and the percentage of splenocytes that were $CD4^+KJ1^+$ was determined. The remaining splenocytes were then spun and resuspended in a volume of HBSS to a concentration of $5x10^6$ $CD4^+KJ1^+$ cells/ml. 200 µl ($1x10^6$) cells were injected into the tail vein of each recipient Balb/c mouse.

### 2.7.3. PMDD immunisation

The abdomen of mice to be immunised was shaved and one cartridge shot (or two, if specified) was administered to each side of the abdomen (Figure 2.10). Helium at a pressure of 500 psi was used to propel the microparticles at high velocity using the Helios gene gun (Bio-Rad), kindly provided by Prof Jeremy Brockes (Dept of Biochemistry, UCL, London, UK). When repeat doses were administered, it was ensured that immunisation sites were non-overlapping.

### 2.7.4. Induction of asthma

In the original model mice were sensitised with 10 µg OVA adsorbed to 2 mg alum in 0.2 ml sterile saline, administered intraperitoneally twice, 14 days apart. In this study the first sensitisation was replaced by the gene gun immunisation schedule described in chapter 7. On days 10, 11 and 12 post-sensitisation asthma was induced by intranasal administration of 50 µg OVA in 50 µl saline (”naive”, “primed” and “treated” groups). The “control” group instead received intranasal saline. 3 days post-induction (15 days post-sensitisation), mice were sacrificed and bronchoalveolar lavage (BAL) fluid, lymph nodes and blood were obtained.

## 2.8. Harvesting of cells

### 2.8.1. Preparation of lymph node cells

On day 3 and/or 5 post-immunisation, inguinal lymph nodes were harvested and homogenised between two sterilised ground-glass microscope slides and cells were suspended in HBSS, counted and resuspended in a volume of complete RMPI (RPMI-1640, Invitrogen-Gibco), to give a cell concentration appropriate to the analysis method(s) used, normally $8x10^6$ cells/ml.

### 2.8.2. Asthma model: Blood, BAL fluid and lymph nodes

Mice were sacrificed by intraperitoneal administration of 200 µl sodium pentobarbital to prevent damage to the aorta and trachea. The skin and tissue under the chin was cut away and the resulting pool of blood, assisted by heart massage, was harvested by pipette.

A hyperdermic needle was used to pierce the trachea. A syringe was used to flush the lungs 5 times with 1 ml aliquots of PBS containing 0.1% BSA and 0.37% EDTA.

The rib cage was then opened and mediastinal lymph nodes harvested.

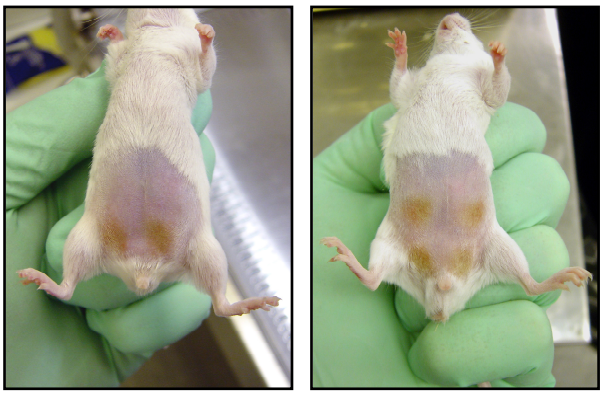


**Figure 2-10. PMDD immunisation.**
The characteristic markings on the skin after the administration of one (left) or two (right) immunisation doses to each side of the abdomen.

## 2.9. Flow cytometry

### 2.9.1. Extracellular antibody staining

After blocking in 10% rabbit serum, lymph node cells or splenocytes were stained with combinations of the following: TriColor-anti-CD4 antibody (Clone CT-CD4, Caltag RM2506), Phycoerythrin-anti-DO11.10 TCR antibody (Clone KJ1.26, Caltag MM7504-3), Phycoerythrin-anti-TNP (isotype control) antibody (Mouse $IgG_2a$, Caltag MG2a04), FITC-anti-CD62L antibody (Caltag RM4301); all at 280 ng per million cells. Cells were then washed in PBS containing 0.5% Bovine Serum Albumin Cells were collected using a Becton Dickinson FACScan™ and analysis was performed using CELLQuest™ (Becton Dickinson) or WinMDI (Joseph Trotter, Scripps Research Institute) software.

### 2.9.2. Intracellular antibody staining

$4x10^6$ lymph node cells were restimulated for 6 h in complete RPMI medium containing 1 μM $OVA_{323-339}$ (ISQAVHAAHAEINEAGR, provided by GlaxoSmithKline) and 10 μg/ml Brefeldin A. Cells were stained extracellularly as above and were fixed and permeablised using the Fix-n-Perm kit (Caltag GAS-003) according to the manufacturer's guidelines. Cells were stained with 5 μl FITC-anti-IL-2 antibody (Immunotech PNIM3017).

### 2.9.3. CFSE labelling

A stock of CFSE was prepared by dissolving lyophilised CFSE (Molecular Probes / Invitrogen) in extra-dry dimethyl sulfoxide (DMSO) to a concentration of 5 mM. The stock was stored airtight at –80°C. After preparation for adoptive transfer as above, splenocytes were resuspended to $3.77x10^6$ cells/ml in HBSS and equilibriated to 37°C in a waterbath (around 15 min). Stock CFSE was added to a final concentration of 5 μM and the labelling reaction was allowed to proceed at 37°C for another 5 min. Cells were washed three times in large volumes of HBSS before resuspending to the appropriate volume for adoptive transfer (see above).

### 2.9.4. Measurement of CFSE-stained T cell proliferation by flow cytometry

The total number of cell divisions of CFSE-stained DO11.10 cells was calculated as follows: Lymphocytes were gated by forward and side-scatter analysis. DO11.10 cells were gated by CD4 and KJ1.26 expression. FL1 fluorescence by these cells was displayed on a histogram (see Figure 2-11 for example). The peak farthest to the right (i.e. highest fluorescence) was considered to contain cells that had not yet divided. Each of the peaks to the left were considered to contain cells that had undergone one more division than the peak on their right. For example, the third peak (M3 on the graph) would contain cells that had undergone two divisions. The number of cells in each peak was divided by $2^X$ where X is the number of divisions for that peak. The figures for each peak were added to give the total number of divisions caused by the treatment. A caveat to interpretation of this data is that, for simplicity, this method assumes zero cell death.

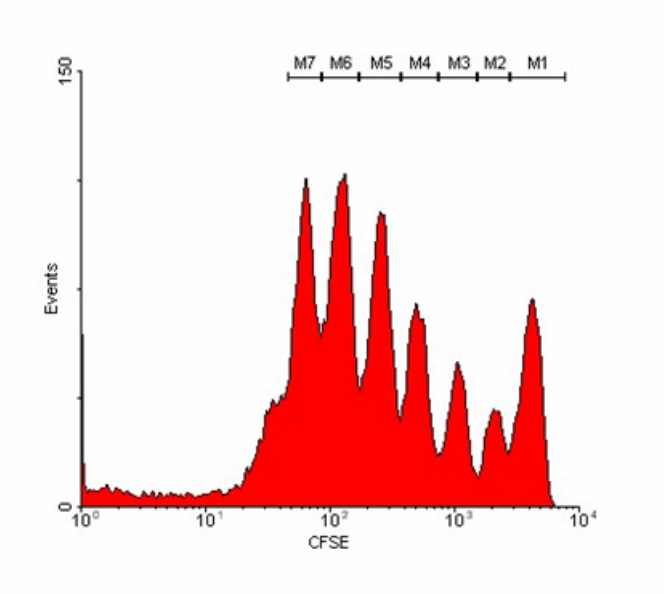



**Figure 2-11. Measurement of CFSE-stained T cell proliferation by flow cytometry.**
A characteristic histogram of CFSE fluorescence by adoptively transferred DO11.10 cells following immunisation. Rightmost peak represents undivided cells; each peak to the left represents cells that have undergone one more division than those represented by the peak on its right. For example, peak M3 represents cells that have undergone two divisions.

## 2.10. Enzyme-linked cytokine assays

### 2.10.1. IL-10 ELISA on supernatant of transfected CHO cells

ELISA plates (Falcon 3915) were coated with 4 μg/ml anti-IL-10 (Pharmingen 554422) in bicarbonate buffer overnight at 4°C. They were then washed 3 times with PBST and nonspecific binding sites were blocked by incubating for at least 2 hours at room temperature with 200μl per well of PBS/1% BSA. Serial dilutions of sample supernatants were added to wells alongside a standard curve of recombinant IL-10 (Pharmingen 550010 or PeproTech EC 210-10) and plates were incubated for 2 hours at 37°C. After washing 3 times with PBST, 2 μg/ml biotinylated anti-IL-10 in PBS/1%BSA was added to each well and incubated for 1 h at room temperature. A further 4 washes was followed by incubation with Streptavidin Peroxidase (Pharmingen 554066) 1:1000 in PBS/1%BSA. After a final 5 washes, plates were developed using o-phenylenediamine dichloride (Sigma P-6912) and read at 450 nm on a U-1500 spectrophotometer (Hitachi) using Revelation software (Dynex Technologies).

### 2.10.2. IgE ELISAs on blood serum of asthma-induced mice

The same procedure as above was performed apart from the following differences: For the total IgE ELISA the coating (capture) antibody used was anti-IgE (Rat $IgG_1$, Pharmingen 553413) and the detecting antibody was biotinylated anti-IgE (Rat $IgG_1$, Pharmingen 553419). The standard curve was built using recombinant IgE (Pharmingen 557079). For the OVA-specific IgE ELISA the same detecting antibody was used but the coating (capture) substance was ovalbumin (20 μg/ml in PBS) rather than antibody.

### 2.10.3. ELISPOT

The day prior to lymph node or spleen harvest, 96-well ELISPOT plates (Millipore, MAIPS45) were coated with 50μl per well of sterile anti-cytokine antibody at 10μg/ml (IL-2, IL-4, IL-10) or 15μg/ml (IFN-g) in HBSS (anti-IL-2 clone JES6-1A12, eBioscience 14-7022; anti-IFN-γ clone R4-6A2, Pharmingen 551216; anti-IL-4 clone 11B11, eBioscience 14-7041; anti-IL-10 clone JES5-16E3, eBioscience 14-7101). Plates were incubated at 4°C overnight. On the day of lymph node harvest, plates were washed three times with 200μl per well of HBSS and nonspecific binding sites were blocked by incubating for at least 2 hours with 200μl per well of complete RMPI. Routinely, 1, 2 and $4x10^5$ lymph node cells were plated per well (2, 4 and $8x10^5$ for IFN-γ) and data was multiplied so as to be presented in spots per million cells. Where coculture ELISPOTs were performed, $5x10^3$ and $1x10^4$ DO11.10 cells per well were cocultured with $4x10^5$, $2x10^5$ and $1x10^5$ splenocytes per well. $OVA_{323-339}$ was dissolved in complete RPMI to give a final concentration in ELISPOT wells of 1μm. Control wells received complete RPMI instead. The total volume in all wells was 200μl. Plates were incubated at 37°C and 5% $CO_2$ for 19h.

Cells were removed from plates by washing in PBS, incubating with 200μl $dH_20$ for 10 mins to lyse remaining cells and washing a further three times with PBS. 50μl of the appropriate biotinylated

antibody (biotin-anti-IL2 clone JES6-5H4, Pharmingen 554426; biotin-anti-IFN-γ clone XMG1.2, Pharmingen 554410; biotin-anti-IL-4 clone BVD6-24G2, Pharmingen 554390; biotin-anti-IL-10 clone JES5-2A5) at 1μg/ml in HBSS was added to the relevant wells and incubated at room temperature with shaking for 2 hours. Plates were washed three times and 50μl streptavidin-alkaline phosphatase conjugate (Caltag SA1008) diluted 1:1000 in HBSS was added per well. After another one hour room temperature incubation with shaking, wells were washed five times. 50μl reagent (Bio-Rad 170-6432) was added to each well and spots were allowed to develop. The reaction was stopped by washing twice with 200μl $dH_20$ per well. Spots were counted a day later using an image analysis system (Autoimmun Diagnostika GmbH, Strassberg, Germany).

For human interferon-γ (h-IFN-γ) ELISpot, used to detect migrating p7.hIFNg-transfected DC, Iscoves medium replaced RMPI for all steps. The spots produced by the h-IFN-γ ELISpot were initially of too low intensity to be reliably counted. Therefore incubation was extended to 45 h.

# 3. Expression of vector constructs

## 3.1. Introduction

As well as confirming the successful construction of the vectors to be used, this chapter examines and validates some of the current knowledge of the mechanism of particle mediated DNA delivery (PMDD or gene gun).

There has been much discussion over the exact mechanism of PMDD immunisation. Most relevant to this chapter is the temporal distribution of antigen expression versus dendritic cell (DC) migration from the skin to the lymph node. Even under control of a promoter as well studied as CMVie variations in the kinetics of expression and antigen presentation are possible. Firstly this novel method of introducing the promoter and gene into the cell may bypass the natural processes which would normally define expression kinetics. Secondly there is the question of which cell or cells are transfected and on which of these is detection of expressed protein dependent. More specifically are DC directly transfected prior to their migration or do they take up antigen from adjacent transfected skin cells (e.g. keratinocytes)?

It has been shown that, under the control of the CMVie promoter, expression of transgene occurs <24 h post-PMDD[(Torres *et al.*, 1997; Porgador *et al.*, 1998; Larregina *et al.*, 2001; Ludwig-Portugall *et al.*, 2004)]. However it is not obvious when expression ceases, not least because the product of each transgene studied will have a different half-life, both within the cell and in the extracellular space. In the case of immunisation, also important is the duration of antigen presentation by any transfected skin DC – partially dependent on transgene expression but also on the stability of antigen-MHC complexes on the cell surface.

PMDD immunisation directly transfects both skin DC and keratinocytes[(Condon *et al.*, 1996; Lu *et al.*, 1996; Porgador *et al.*, 1998)]. The number of keratinocytes in skin greatly exceeds that of DC and it is likely that the proportion of transfected cells follows this ratio. However the evidence suggests that skin DC predominantly present antigen from direct transfection as opposed to antigen from surrounding transfected cells[(Porgador *et al.*, 1998; Timares *et al.*, 2003)]. It has been shown that directly transfected DC migrate to the lymph node and that they are vital for immunisation[(Morita *et al.*, 2001; Ross *et al.*, 2003)]. Although the precise characteristics of migration and transgene expression may be dependent on the specific model, this information suggests that, whereas transgene expression in the skin occurs in all cell types, the majority of expressed protein in draining lymph nodes will originate from directly transfected, migrated skin DC. This has implication for the detection of transgene expression *in vivo*.

## 3.2. Expression of IL-10 *in vitro*

### 3.2.1. Transfection of Chinese Hamster Ovary cells with p7.IL10

To establish the functionality of the p7.IL10 vector, expression of IL-10 was tested following *in vitro* transfection of Chinese Hamster Ovary (CHO) cells. CHO cells were transfected with 1µg p7.10. 48h

later supernatants were harvested and IL-10 expression analysed by ELISA (Figure 3-1). To detect any possible interference by p7.OVA on p7.IL10, the same procedure was performed using a mixture of 1μg of each vector. As a negative control, a third transfection was performed using p7.OVA alone.

Transfection with p7.IL10 alone produced a detectable quantity of IL-10. This was not quantified in these experiments since the objective was only to confirm that the vector was functional. There was no background production or cross-reaction with other molecules as evidenced by the cells transfected with p7.OVA alone. p7.OVA did not interfere with transfection or expression of p7.IL10; when transfected together, the level of IL-10 production was indistinguishable from that of p7.IL10 alone.

## 3.3. Expression of OVA *in vitro*

### 3.3.1. In vitro gene gun of MC57 fibrosarcoma cells with p7.OVA

In order to test the expression of p7.OVA it was necessary to perform an experiment similar to that in 3.2.1. In addition, it was important to determine the ability of DNA bound to gold particles in the gene gun system to be expressed. Therefore an *in vitro* gene gun system was developed (see Materials and Methods).

Gene gun cartridges containing low (0.05 μg), medium (0.1 μg) and high (0.5 μg) doses of p7.OVA and/or p7.IL10 were prepared. Negative control cartridges containing only empty p7 were also prepared. $4x10^6$ MC57 cells were transfected with one of these three cartridges. Recombinant IL-10 was added to relevant wells to a final concentration of 20 ng/ml. Cells were incubated for 24 h after which time wells were lysed with 200 μl $H_20$.

Cell lysates were analysed by Western blot (Figure 3-2). Ovalbumin, with a molecular weight of around 43kDa, was observed to form a double-band roughly midway between the 30kDa and 50kDa molecular weight markers. This band was absent in lane 1, containing lysate from empty p7-transfected cells. Lanes 2, 3 and 4 show an increase in band intensity corresponding to an increase in p7.OVA dose. Into the highest dose of p7.OVA (0.5 μg) was titrated a range of p7.IL10 doses. Lanes 5 and 6 show that low and medium doses of p7.IL10 have no detectable effect on the expression of p7.OVA. However the highest dose of p7.IL10 was observed to cause a slight decrease in the concentration of OVA in the cell lysate (lane 7). To estimate the relative concentration of OVA as compared to cells transfected with p7.OVA alone, the band intensity of lane 7 was compared to that of lanes 2-4. From this comparison it is evident that cells transfected with 0.5 μg of both p7.OVA and p7.IL10, although expressing p7.OVA to a lesser degree than cells transfected with 0.5 μg p7.OVA alone, expressed p7.OVA to a greater degree than cells transfected with 0.1 μg p7.OVA.

To distinguish between plasmid competition and a specific effect on expression by synthesised IL-10, recombinant murine IL-10 (20 ng/ml) was added to cells transfected with low and high doses of p7.OVA (lanes 8 and 9, respectively). Band intensity was similar, if not identical, between lanes 2 and 8 and between lanes 4 and 9. Thus expression of p7.OVA, at least by MC57 cells, was not affected by

the presence of IL-10 protein. Any reduction in expression seen in lane 7 must therefore be due to competition on the nucleic acid level.

### 3.3.2. *In vitro* gene gun of bone marrow-derived dendritic cells – p7 constructs

To extend the possibilities of *in vitro* PMDD and to further validate the expression of the p7.OVA vector the standard gene gun barrel was modified (see Materials and Methods). Whereas the standard equipment successfully transfected MC57 fibrosarcoma cells (3.3.1), it was not efficient at transfecting bone marrow-derived dendritic cells (BMDC); it caused extensive cell death, especially in the central 'dead' zone of the blast. The modified barrel diffused the power of the gene gun blast and spread the gold particles more evenly over the blast area (see 2.4.3). This made it possible to examine the expression of p7.OVA in cultured DC.

BMDC were transfected with 0.5µg p7.OVA with or without 0.5µg p7.IL10. Controls were either transfected with empty p7 or were untransfected. Recombinant murine IL-10 (20 ng/ml) was added to the relevant wells. Cells were incubated for 24 h, lysed in 200 µl $H_20$. and lysates analysed by Western blot (Figure 3-3).

Blot A compares various gene gun treatments with untransfected DC. The distinct double-band is seen only in lysates of cells that received p7.OVA. Whereas Figure 3-2 indicates a small inhibition of p7.OVA by p7.IL10, Figure 3-3 suggests that this may not be the case in DC: No difference was observed between cells that received p7.OVA alone and those that received p7.OVA + p7.IL10. Similar to expression in MC57 cells, the presence of recombinant murine IL-10 had no effect on the expression of p7.OVA.

The accompanying Coomassie stain (B) of the polyacrylamide gel used shows equal loading in each lane. In this case it is particularly important as cells that have been bombarded by PMDD are being compared to those which have not.

To confirm that the double-band seen in the previous Western blots was indeed expressed ovalbumin, lysates of transfected DC were run alongside a solution of ovalbumin. Blot C shows that the double-band present in the lysate of p7.OVA-transfected DC, and absent in untransfected and p7-transfected DC, is the same size as ovalbumin.

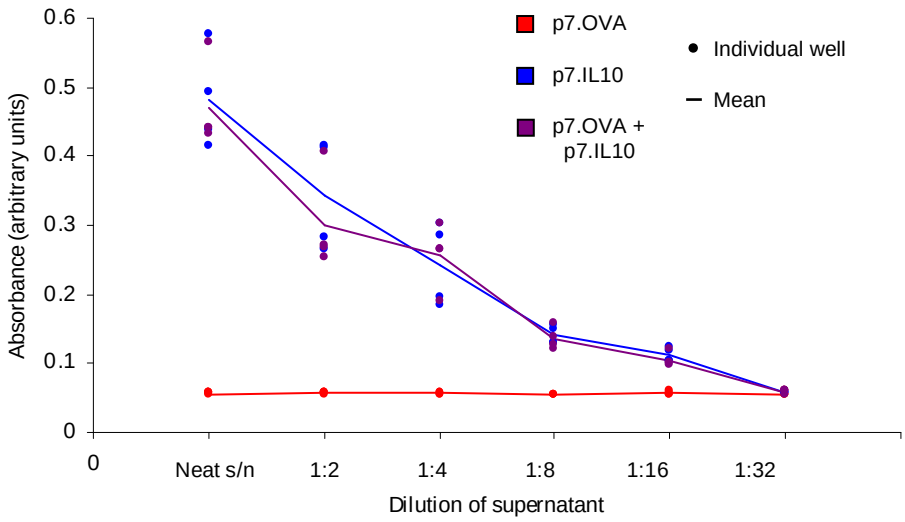



**Figure 3-1. *In vitro* expression of IL-10.**
CHO cells were transfected with 0.5 µg p7.IL10, 0.5 µg p7.OVA or both. After 48 h incubation supernatants were analysed by IL-10 ELISA. Data are the mean of two independent experiments; each dot represents an individual well.

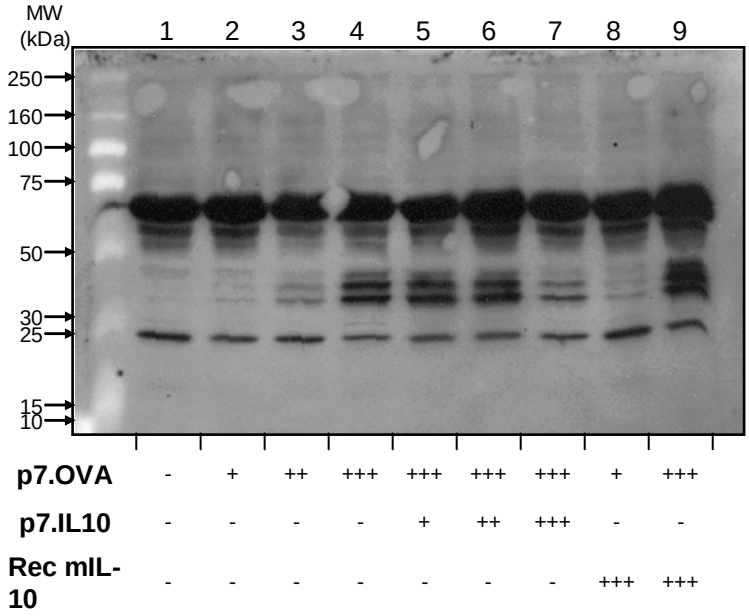



**Figure 3-2. *In vitro* expression of OVA using PMDD.**
A droplet containing 4x10$^6$ MC57 cells was transfected with one gene gun cartridge using compressed helium at 275 psi. Each cartridge contained a combination of high (0.5 µg; +++), medium (0.1 µg; ++), low (0.05 µg; +) or no (–) p7.OVA and/or p7.IL10. Cells were incubated for 24 h in the presence (+++) or absence (–) of 20 ng/ml recombinant murine IL-10. Cells lysates were examined by Western blot.

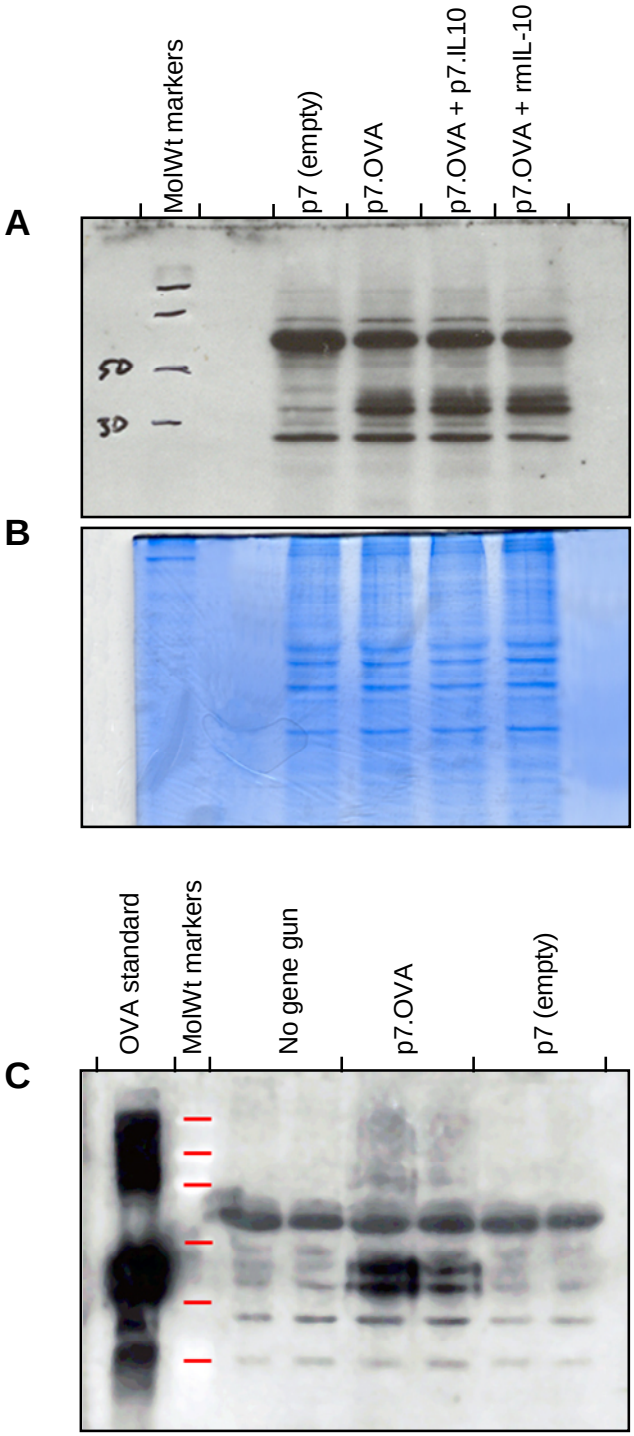



**Figure 3-3. *In vitro* transfection of bone marrow-derived DC using PMDD.**
$1x10^6$ bone marrow-derived DC were transfected, using modified gene gun equipment, with 0.5 µg p7.OVA with or without 0.5 µg p7.IL10. Controls were either transfected with empty p7 vector alone or were untransfected. Cells were incubated for 24 h and lysed in 200 µl $H_20$. Lysates were run on a 12% polyacrylamide gel, transferred onto nitrocellulose membrane and probed with a polyclonal anti-OVA antisera. A) Western blot comparing expression of p7.OVA in the presence or absence of p7.IL10 or recombinant murine IL-10. B) Coomassie stain of the polyacrylamide gel to examine lane loading. C) Western blot to compare the band present in lysates of p7.OVA-transfected cells with a standard solution of ovalbumin (3 pg).

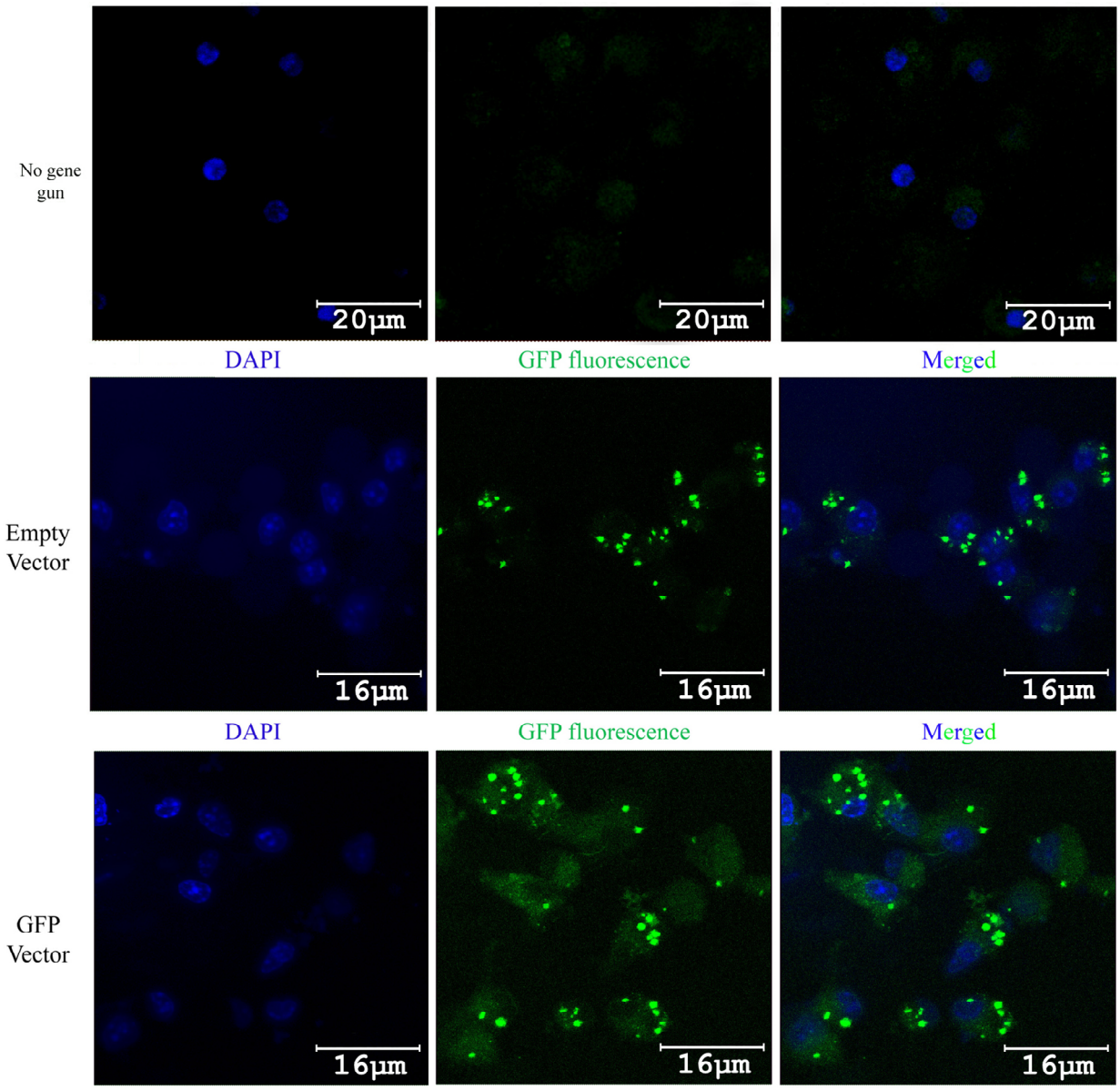



**Figure 3-4. Fluorescence microscopic analysis of *in vitro* PMDD system using a GFP-encoding vector.**
1x10$^6$ BMDC were left untransfected (top row) or transfected, using modified gene gun equipment, with empty p7 (middle row) or with p7.GFP (bottom row) Cells were incubated for 24 h and then, bound to coverslips, were washed and mounted onto microscope slides using Vectashield mounting medium containing 1.5 µg/ml DAPI. Cells were studied by confocal microscopy. Blue fluorescence delineates DAPI-stained cell nuclei (left column). Green fluorescence identifies gene gun beads and GFP (middle column). Merged colours produce a clear picture of GFP expression/nonexpression in cells (right column).

### 3.3.3. *In vitro* gene gun of bone marrow-derived DC – GFP constructs

To follow expression of genes administered by PMDD at the single-cell level and to potentially enable future tracking of *in vivo* transfected cells, expression of a GFP-encoding vector (pHR-SIN-cPPT-SE[(Demaison *et al.*, 2002)], kindly provided by M. Collins) was examined following *in vitro* PMDD. Transfected DC were incubated for 24 h at which time they were stained, mounted and examined by confocal microscopy (Figure 3-4).

The nuclei of all cells were highly visible due to the DAPI stain. In cells which had received PMDD of empty p7 vector, the gold beads were visible as punctate green fluorescence that was not present in DC that had not received PMDD. These points were also present in cells that had received the GFP-encoding vector. However in this case green fluorescence was seen throughout the cytoplasm, suggesting expression of GFP. Further work would probably be required to prove absolutely that the beads were inside the cells and not merely associated with the membrane.

## 3.4. Expression of OVA *in vivo*

### 3.4.1. Detection of expression of p7.OVA by Western blot

Several attempts were made to measure OVA in homogenates of transfected skin tissue and draining lymph nodes by Western blot (Figure 3-5). In skin homogenates, occasional blots suggested detectable levels of the protein but results were not reproducible. The problem was confounded by cross-reactivity of the polyclonal anti-OVA antibody with a component of skin. Figure 3-5a demonstrates this cross-reactivity: Lanes containing homogenates of untransfected and p7-transfected skin display two bands, one of approximately the same molecular weight as OVA and the other of approximately 30 kDa. Despite increased intensity in the p7.OVA-transfected skin, it was not possible to confidently detect OVA.

Homogenates of lymph nodes from the same mice were also analysed for OVA expression. In a similar way to skin samples, occasional blots suggested the presence of detectable amounts of ovalbumin but the majority of attempts were unable to detect the protein (Figure 3-5b). Therefore expression of p7.OVA in vivo could not be confirmed by Western blot.

### 3.4.2. Detection of plasmid expression by antigen presentation ELISpot

An indirect method to demonstrate the expression of p7.OVA *in vivo* was devised, the aim being to detect the presence of transfected skin DC in the draining lymph node via antigen presentation ( Figure 3-6). p7.OVA or empty p7 were administered by PMDD. In this case mice received 4 gene gun shots, totalling 1 μg vector, in order to maximise the possibility of detecting transfected DC. 24 h following PMDD lymph node cells were cocultured with pre-primed DO11.10 cells on an anti-IL-2-coated ELISpot plate and incubated for 24 h.

The number of spots detected was extremely small: Cells from mice that had received p7.OVA produced an average of 15 spots per lymph node while those from mice immunised with empty p7 produced around 5-10 spots. However the number of spots was similar in the presence or absence of added DO11.10 cells, suggesting that they may have been due to background production of IL-2 by the lymph node cells. These experiments did not convincingly demonstrate antigen presentation by DC from immunised mice.

### 3.4.3. Detection of plasmid expression by direct ELISpot

A more direct method to detect plasmid expression *in vivo* (or *ex vivo*) was tested in order to provide more accurate enumeration of transfected, migrating cells. To prevent the cross-reactivity of anti-OVA detection antibody that was seen in Western blots and to avoid confusion with endogenous production of protein, neither p7.OVA nor p7.IL10 were used for this purpose. Instead the human interferon-γ (hIFN-γ) cDNA was cloned into the p7 vector and a standard capture-detection antibody pair was used in an ELISpot to detect hIFN-γ-expressing cells in the lymph node. Mice received two shots of either a 'normal' (0.5 μg) or high (1.0 μg) dose of p7.hIFNg. 6 h and 21 h post-PMDD a 45 h ELISpot was performed on cells from draining inguinal lymph nodes.

At 6 h post-PMDD, an average of <1 spot per lymph node was detected. Because all three groups produced a similar number of spots, this was considered background. After 21 h both low and high doses of p7.hIFNg showed a increase in the number of hIFN-γ-producing cells with an average of 6.6 and 7.1 spots per lymph node, respectively. Thus around 6 detectable transfected cells migrated from the skin to each lymph node. A doubling of the number of plasmid copies per gold particle had little effect on the number of cells detected.

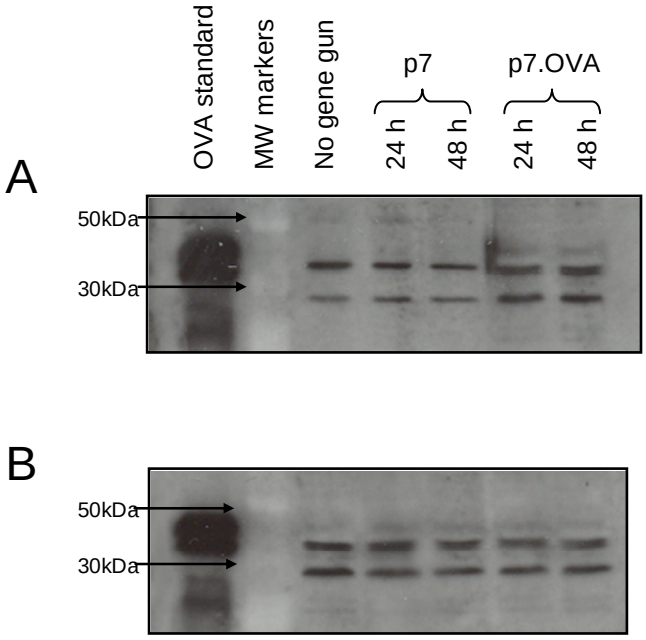



**Figure 3-5. *In vivo* PMDD – detection of OVA expression by Western blot.**
Mice either received PMDD of 0.5 µg p7.OVA or empty p7 or did not receive PMDD. After 24 or 48 h skin from the gene gun site (A) or draining lymph nodes (B) were harvested, homogenised and analysed by Western blot. Each group consisted of three mice.

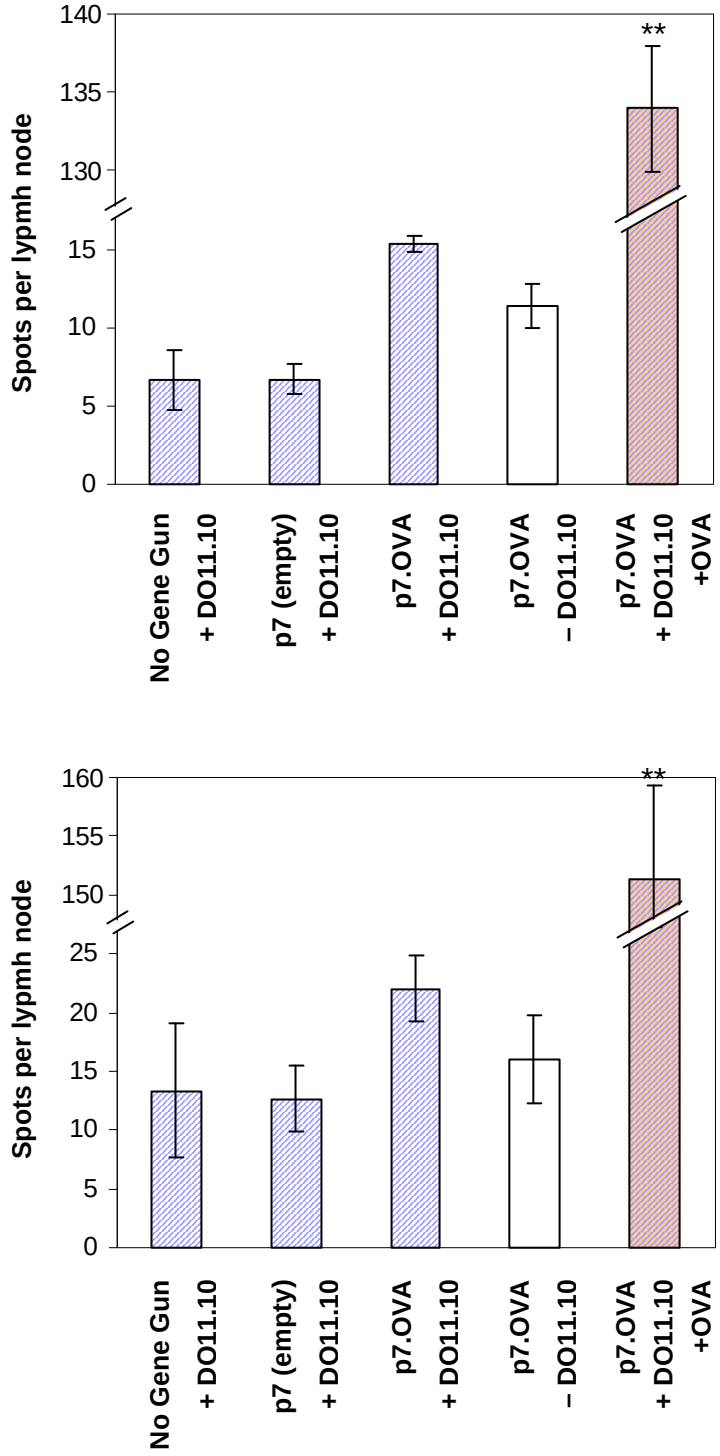



**Figure 3-6. Detection of migrating transduced skin cells in the lymph node by antigen presentation ELISpot.**

Mice (3 per group) either received PMDD of p7.OVA or empty p7 or did not receive PMDD. PMDD consisted of 4 gene gun shots of 0.5 µg vector to maximise the number of transduced cells. After 24 h draining lymph nodes were harvested and cell suspensions prepared.

$1x10^6$ lymph node cells were cocultured with $5x10^4$ antigen-experienced DO11.10 T cells in an IL-2 ELISpot. Control wells contained lymph node cells with no DO11.10 cells or contained exogenous $OVA_{323-339}$.

Figures are adjusted to spots per lymph node. Graphs represent independent experiements. Each bar represents a triplicate ELISpot well.

** $p<0.01$ by one-way ANOVA

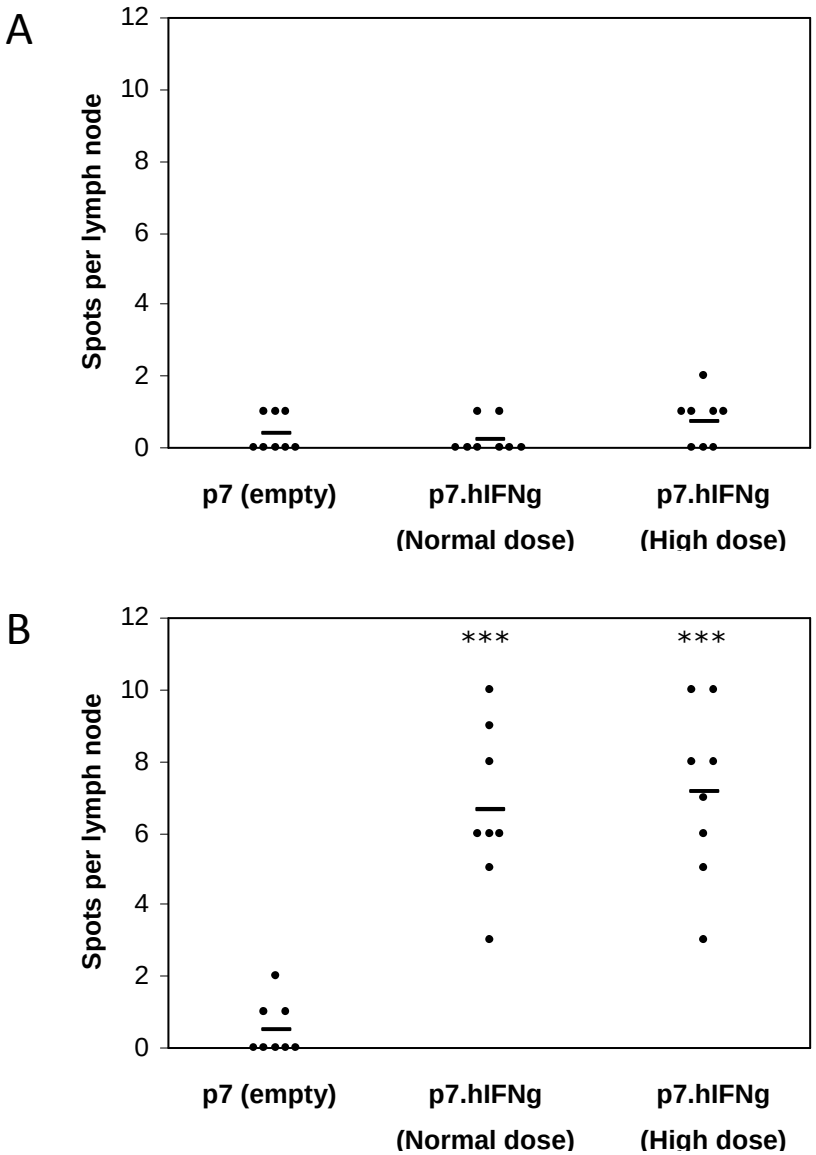



**Figure 3-7. Detection of migrating transduced skin cells in the lymph node by human IFN-γ ELISpot.**
Mice received 2 shots of either empty p7, a 'normal' dose of p7.hIFNg (0.5 µg) or a 'high' dose of p7.hIFNg (1.0 µg). Draining inguinal lymph nodes were harvested either 6 h (panel A) or 21 h (panel B) post-PMDD and cultured alone for 45 h in an anti-hIFN-γ-coated ELISpot plate. Each point represents an individual mouse. Bars represent the mean for each group.
***p<0.001

## 3.5. Discussion

### 3.5.1. Expression of vectors *in vitro*

*In vitro* transfection of both p7.IL10 and p7.OVA into CHO cells and MC57 fibrosarcoma cells resulted in reliable expression of IL-10 and OVA, respectively. No significant inhibition or competition was detected between the vectors when transfected together.

This expression study required the development of a novel technique for the transfection of DC *in vitro*. Whether it is generally more efficient than other techniques remains to be seen but it does provide a more appropriate means by which aspects of PMDD immunisation can be studied *in vitro*. Western blot analysis of p7.OVA-transfected bone marrow-derived DC (BMDC) provided evidence of successful transfection but the system was not entirely reliable. Although the polyclonal detection antibody used was remarkably sensitive (bands contained <3pg OVA), background noise and multiple bands were common problems.

An antibody-free technique was developed to circumvent these problems: BMDC were transfected with vector encoding GFP. It came as some surprise that the gold beads used to transfect the DC were themselves visible through the green channel. However, initial concerns that the green fluorescence observed in the cytoplasm of GFP-transfected cells was merely diffusion of light from the gold beads were laid to rest upon observing the p7-transfected cells which contained the beads but whose cytoplasm did not fluoresce. Unfortunately one of the reasons for developing the GFP gene-gun system was to track transfected cells in vivo, possibly by flow cytometry. Even if green-fluorescing cells were detectable, those containing beads and GFP may well be indistinguishable from those containing beads alone. Nevertheless, reliable detection of green fluorescent cells in the p7.GFP-transfected group was achieved, demonstrating the efficiency of this novel transfection technique.

### 3.5.2. Expression of vectors *in vivo*

Similar problems to those above were encountered when Western blot analysis of transfected skin and its draining lymph nodes was performed. Cross-reactivity with an endogenous component of skin prevented the identification of expression of p7.OVA in that tissue. This component was not present in draining lymph nodes but reliable detection by Western blot was nevertheless not possible.

Previous studies have also found that detection of protein in migratory skin DC was problematic. In one model expression, under control of the CMVie promoter, occurred 12-24 h post-PMDD whereas collection of a useful number of migratory DC required 48 h[(Larregina *et al.*, 2001)]. By this time expression was no longer detectable. It is possible, therefore, that the kinetics of expression versus those of migration could have contributed to the difficulty of detecting OVA expression *in vivo*.

The small number of transfected skin DC that appear in the lymph node make detection by flow cytometry very difficult[(Akbari *et al.*, 1999)]. Using β-gal as the transgene, Porgador *et al*. were able to count, histologically, 10-20 transgene-expressing cells per lymph node per gene gun shot[(Porgador *et al.*, 1998)]. A more elaborate β-gal reporter system was developed by Garg *et al*., using flow cytometry to identify

100 times more transfected DC in the lymph node than were counted by Porgador *et al*[(Garg et al., 2003)]. A key difference between these studies is that the latter involves permanent tagging of transfected DC whilst the former relies on continued expression of transfected gene. Therefore transfected DC can continue to be identified at later timepoints. Indeed the number of transfected DC in the lymph node continued to increase for 2.5 days; the earlier study only examined migration after 1 day.

Despite the existence of these techniques, the potential difficulty in distinguishing p7.GFP-transfected cells by flow cytometry (described in 3.5.1) led to the decision to use two ELISpot assays to detect transgene expression in the draining inguinal lymph nodes. Direct detection of human IFN-γ showed the presence of 5-10 p7.hIFNg-transfected cells per lymph node 21 h post-PMDD. Although not identical, this number is comparable to that determined by Porgador *et al*. Unfortunately, the antigen presentation ELISpot could not confirm the presence of transfected skin cells in the lymph node. Since this study was performed a similar technique, detecting IFN-γ produced by antigen-specific $CD8^+$ T cells, was successfully developed[(Dzutsev *et al.*, 2008)]. Therefore it is possible that future refinement of our antigen presentation ELISpot might successfully detect antigen-transfected skin cells that have migrated to the lymph node.

Despite this low number of directly transfected DC we know that keratinocytes are transfected during PMDD and might have hypothesised that untransfected DC will also obtain OVA from them. The data did not provide any evidence for an extensive indirect antigen processing/presentation pathway in this model.

Although the number of directly transfected DC in the lymph node is low, a measurable immune response is mounted. This is also seen elsewhere and demonstrates the potent immunostumulatory ability of DC[(Akbari *et al.*, 1999)].

In conclusion the data confirms that the vectors to be used for PMDD immunisation can drive expression of their inserted gene in both conventional lipid-based, and novel PMDD-based, *in vitro* transfection systems. The data also shows that these vectors are not mutually inhibitory. It may also be suggested that OVA is expressed in the skin of mice transfected with p7.OVA.

Based on this data and the many previous reports of gene gun immunisation it was decided to proceed with the study of the immune response to p7.OVA and the effect on this response of p7.IL10.

# 4. Characterisation of the Adoptive Transfer Model

## 4.1. Introduction

Although the ultimate aim of immunology is to understand the normal immune system of an animal, genetically modified animal models have proved useful in doing so. The DO11.10 transgenic α/β T cell receptor (TCR) recognises the $OVA_{323-339}$ epitope of OVA presented on the $I\text{-}A^d$ class II MHC molecule and can be identified using the KJ1.26 clonotypic antibody[(Murphy *et al.*, 1990)]. T cells expressing this TCR can be distinguished using the same antibody. Adoptive transfer of T cells from DO11.10 transgenic mice into synergeic Balb/c recipients allows the study of the transgenic subpopulation in the context of a wild-type general T cell population[(Kearney *et al.*, 1994; Pape *et al.*, 1997)]. This convenient identification of DO11.10 transgenic cells and their inevitable dilution into the host population eliminate the need for their purification prior to transfer. This reduces potential artifactual modification due to ex vivo manipulation.

The overall aim of this study is to examine the effect of IL-10 on PMDD immunisation, particularly to determine if its inclusion alongside antigen could induce antigen-specific tolerance. In order to facilitate this, the adoptive transfer model to be used was characterised.

## 4.2. Results

### 4.2.1. DO11.10 cells can be identified in DO11.10 spleen by CD4 and KJ1.26 expression

Splenocytes from Balb/c or DO11.10 were stained with anti-CD4-TC and anti-KJ1.26-PE. Flow cytometric analysis identified a distinct population of $CD4^+KJ1.26^+$ cells that were present in DO11.10 but not Balb/c spleen (Figure 4-1). This population routinely comprised 12%-14% of total DO11.10 splenocytes. $CD4^+KJ1.26^+$ cells from DO11.10 mice or recipient Balb/c mice will routinely be refered to as DO11.10 cells.

### 4.2.2. After adoptive transfer into a Balb/c host, DO11.10 cells can be recovered and identified

As described in Materials and Methods, spleen cells from DO11.10 mice containing $1x10^6$ DO11.10 cells were adoptively transferred into naïve Balb/c recipients. 72 h later inguinal lymph nodes were harvested and lymph node cells stained with anti-CD4-TC and anti-KJ1.26-PE. Flow cytometric analysis identified the DO11.10 cells as a distinct $CD4^+KJ1.26^+$ population that was absent in the lymph nodes of Balb/c mice that did not receive the transfer (Figure 4-2). This population routinely comprised 0.25%-1.0% of lymph node cells.

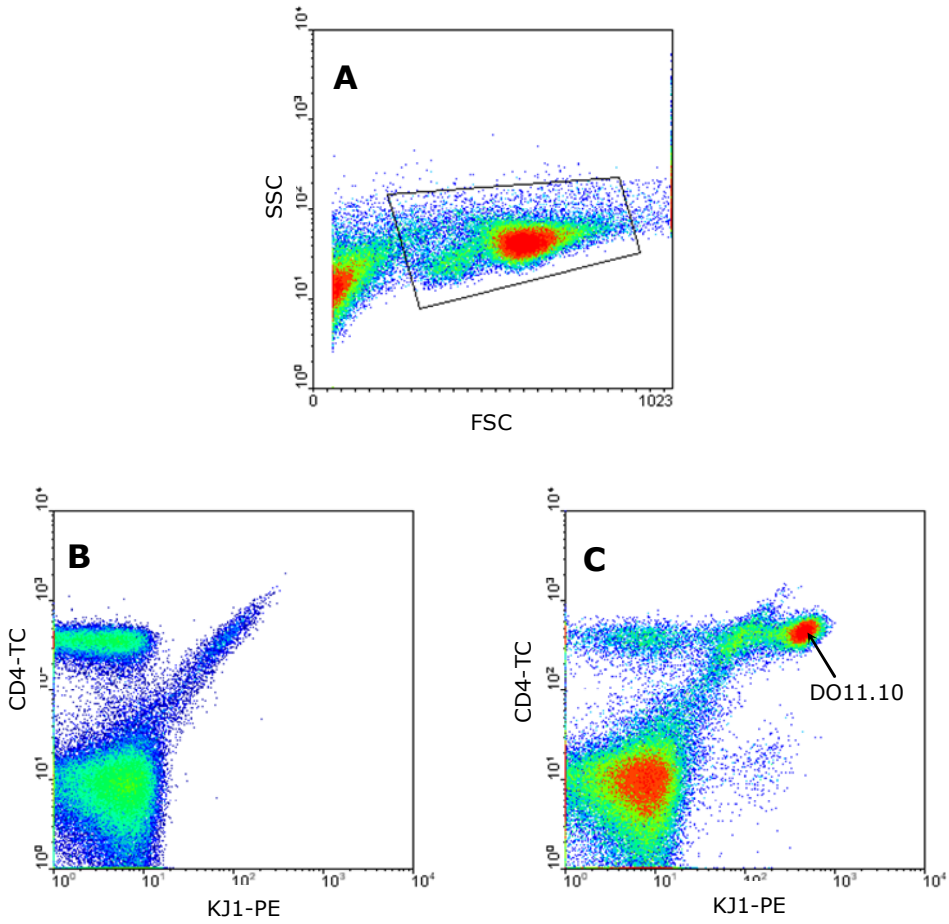



**Figure 4-1. DO11.10 cells can be identified by flow cytometry.**
Splenocytes from Balb/c or DO11.10 mice were stained with anti-CD4 and anti-KJ1.26 (clonotypic TCR). Lymphocytes were gated by forward- (FSC) and side- (SSC) scatter profile (A). Balb/c (B) and DO11.10 (C) lymphocytes were analysed for their CD4 and clonotypic TCR expression. DO11.10 cells were identified by their $CD4^{+}KJ1.26^{+}$ phenotype. Plots are representative of at least 40 independent experiments.

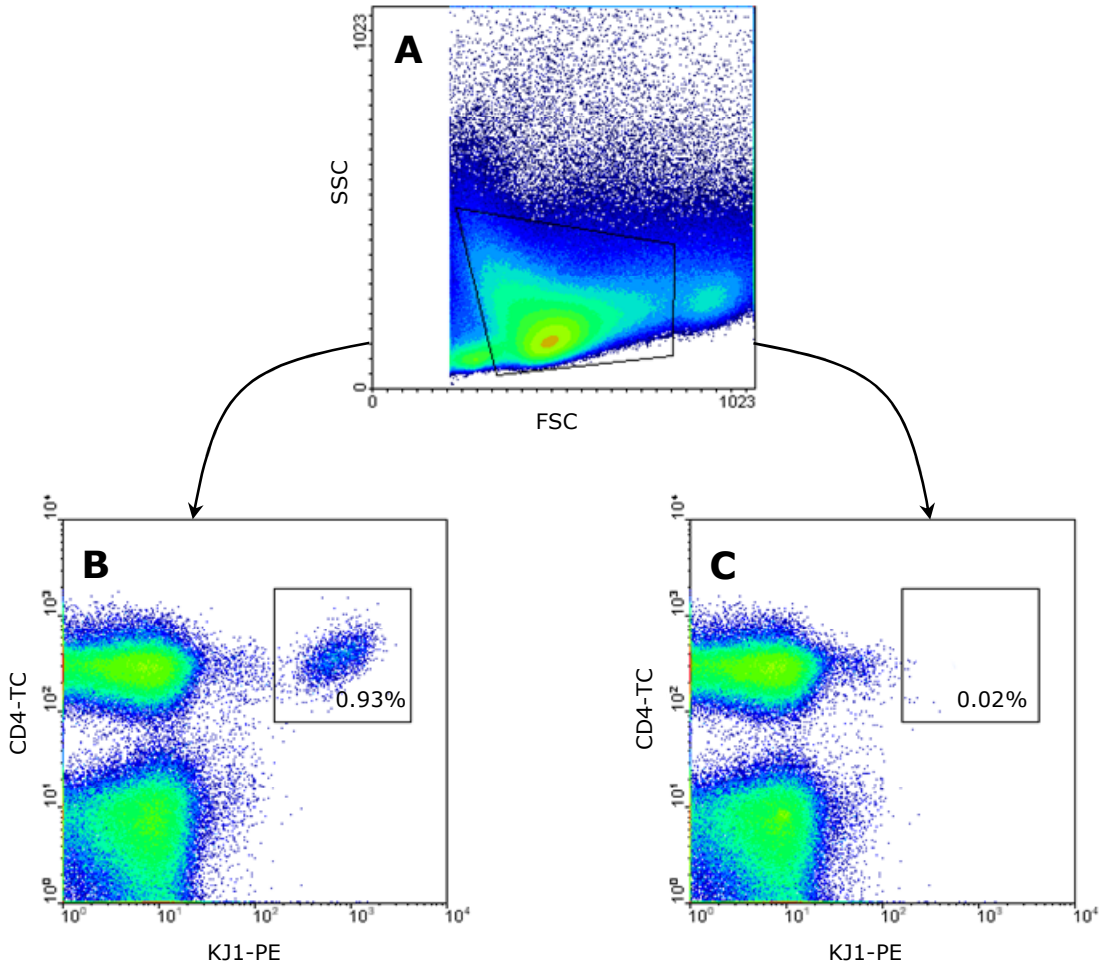



**Figure 4-2. Following adoptive transfer, DO11.10 cells can be identified in lymph nodes.**
1x10$^6$ DO11.10 cells were (B) or were not (C) adoptively transferred into a Balb/c recipient. 96h later, inguinal lymph nodes were harvested and cell suspensions stained with anti-CD4-TC and anti-KJ1.26-PE. Lymphocytes were gated by forward- (FSC) and side- (SSC) scatter profile (A) and DO11.10 cells identified by their CD4$^+$KJ1.26$^+$ phenotype (B and C). Data are representative of at least 40 independent experiments.

### 4.2.3. PMDD of p7.OVA increases the proportion of transferred DO11.10 in draining lymph nodes

$1x10^6$ DO11.10 cells were adoptively transferred into Balb/c recipients. 24 h later the recipient mice were immunised by PMDD with 1 μg p7.OVA or 1 μg empty p7 vector. At various timepoints the draining inguinal lymph nodes were harvested and lymph node cells stained with anti-CD4-TC and anti-KJ1.26-PE. Flow cytometric analysis showed the number of DO11.10 cells in the draining lymph nodes increased dramatically following immunisation with p7.OVA (Figure 4-3). This increase could first be detected 72 h after immunisation and peaked at 96 h post-immunisation. During this time the proportion of $CD4^+KJ1.26^+$ cells roughly doubled every 24 h. Although this effect was not seen following PMDD of p7, a small, transient increase in $CD4^+KJ1.26^+$ cells was observed; seemingly a result of antigen-nonspecific effects of the gene gun. In this case the number of $CD4^+KJ1.26^+$ cells peaked between 72 h and 96 h post-immunisation.

### 4.2.4. PMDD-induced increase in DO11.10 cells is at least partially due to proliferation, not only migration

The increased number of T cells in draining lymph nodes following PMDD immunisation, could be due to antigen-specific T cell proliferation and/or antigen-nonspecific immigration (see discussion). To examine this further a timecourse experiment, similar to that in 0, was performed. In this case, however, splenocytes were stained with carboxyfluoroscein succinimidyl ester (CFSE) prior to adoptive transfer and an additional control group was introduced which received no immunisation at all. 72 h post-immunisation (or 96 h post-adoptive transfer, in the case of the unimmunised group) draining inguinal lymph nodes were harvested and stained with anti-CD4-TC and anti-KJ1.26-PE. As seen in Figure 4-3, immunisation with p7.OVA caused the proportion of DO11.10 in the draining lymph nodes to increase to around 50,000 per million over 72 h and to remain for at least a further 48 h (Figure 4-4a). Also previously observed, immunisation with empty p7 resulted in a transient increase in the $CD4^+KJ1.26^+$ population. The additional control group confirmed that this increase was specific to mice that received PMDD: No increase was seen in mice that did not receive PMDD.

At the 72 h timepoint, flow cytometric analysis was extended to measure the number of cell divisions made by each group (Figure 4-4b). Neither the group that received no gene gun nor that which was immunised with empty p7 exhibited DO11.10 cell division. Significant cell division was seen, however, in the group immunised with p7.OVA. The lack of cell division in either of the first two groups confirms that the increase in DO11.10 cell number in the draining lymph node following immunisation with empty p7 is due to increased net immigration and not to proliferation.

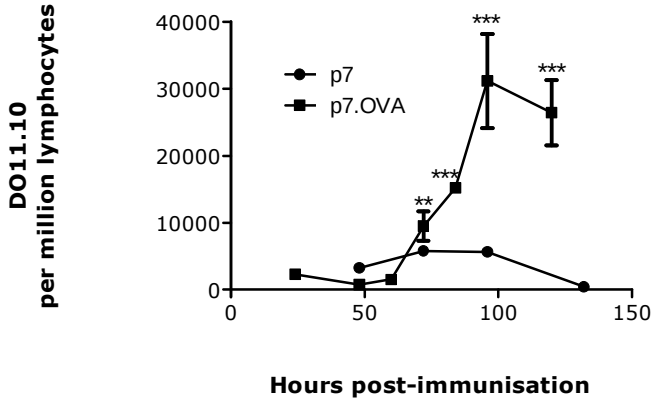



**Figure 4-3. The proportion of DO11.10 cells in the draining lymph nodes increases in response to immunisation with p7.OVA.**
Following adoptive transfer of DO11.10 cells, recipient mice (3 per group) were immunised with 1μg empty p7 or with 1μg p7.OVA. At various points post-immunisation mice were sacrificed and draining lymph nodes harvested. Lymph node cells were stained with anti-CD4 and anti-KJ1.26. DO11.10 cells, identified as $CD4^+KJ1^+$, were enumerated by flow cytometry. Data are the means of four independent experiments.
*** $p<0.001$; ** $p<0.01$ by t-test as compared to p7 control at same timepoint

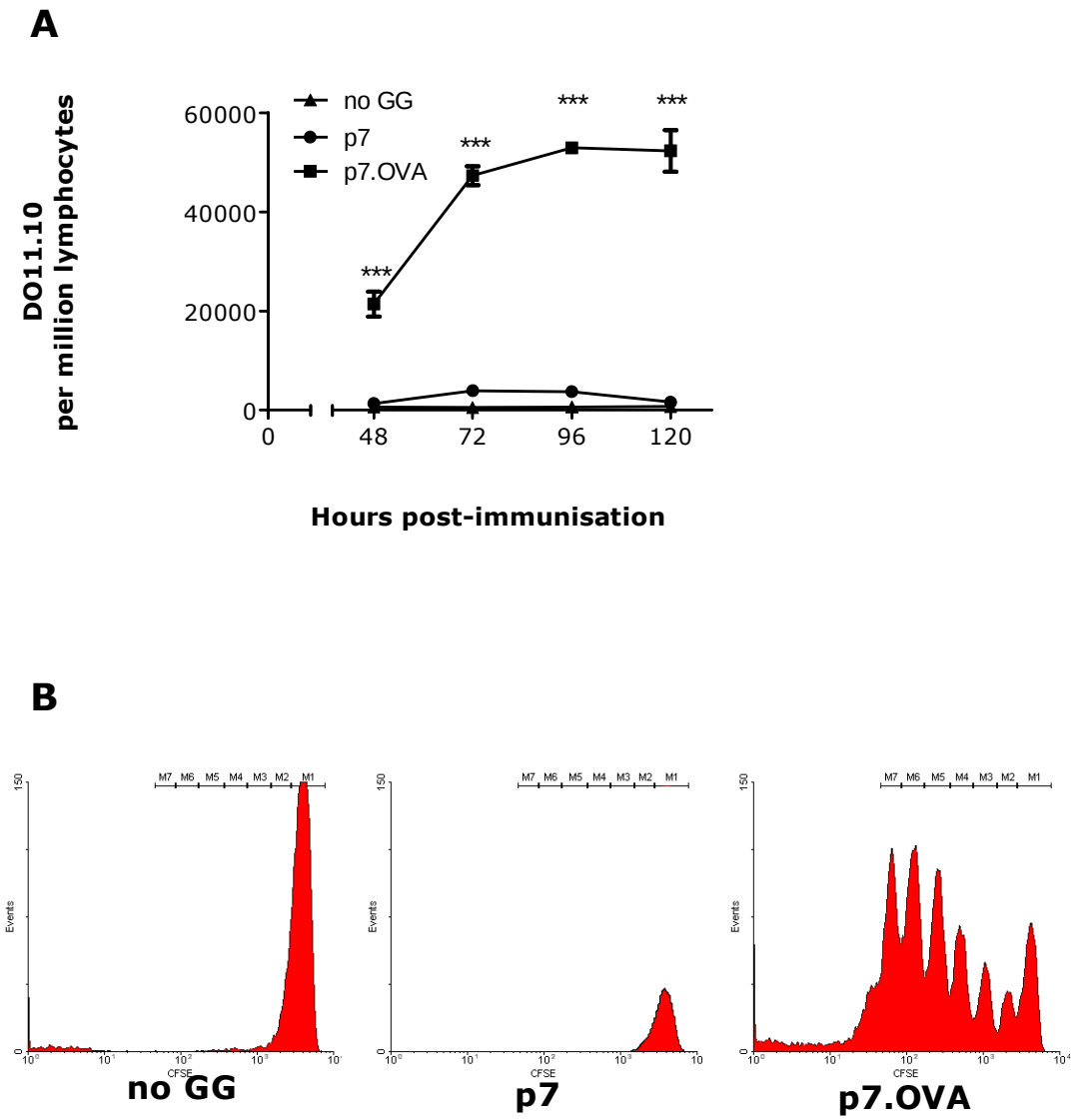



**Figure 4-4. DO11.10 expansion in response to p7.OVA is due to proliferation.**

DO11.10 cells were stained with 5 μM CFSE and $1x10^6$ adoptively transferred into recipient mice. 24 h post-transfer recipient mice (15 per group) were immunised with 1μg empty p7 or with 1μg p7.OVA or were left unimmunised. A) At various timepoints post-immunisation mice (3 per timepoint except 72 h; see below) were sacrificed and draining lymph nodes harvested. Lymph node cells were stained with anti-CD4 and anti-KJ1.26. DO11.10 cells, identified as $CD4^+KJ1^+$, were enumerated by flow cytometry. Data are the means of four independent experiments. B) At the 72 h timepoint an additional 3 mice per group were sacrificed and CFSE fluoresence on gated $CD4^+KJ1^+$ DO11.10 cells examined.

*** $p<0.001$ by t-test as compared to p7 control for same timepoint

### 4.2.5. The increase in number of DO11.10 cells in draining lymph nodes is dose-dependent

To understand the relationship between the quantity of antigen-encoding plasmid and the response to antigen the experiment was performed with a range of p7.OVA doses. As explained in detail in the Materials & Methods chapter, all changes to PMDD immunisations ensured a consistent total DNA load per cartridge by buffering with empty p7 vector.

An initial experiment was carried out to assess the viability of $^{3}$H-thymidine incorporation and IL-2 ELISpot as techniques to measure T cell activity (Figure 4-5). Both techniques showed similar p7.OVA dose-dependent increases in T cell activity. This similarity and the already established link between IL-2 expression and T cell proliferation suggest IL-2 ELISpot is, in this model, a suitable indicator of the proliferative ability of T cells. $^{3}$H-thymidine incorporation assays were therefore discontinued.

A series of more extensive dose-response studies were carried out. During this time the loading of the gene gun cartridges was adjusted for the purpose of consistency between these and future studies which would employ the addition of additional plasmids. The maximal dose of p7.OVA began as 1 μg/mouse (0.5 μg/cartridge) and ended as 0.5 μg/mouse (0.25 μg/cartridge). This provided data from a broad range of doses as is displayed in Figure 4-6.

Empty p7 alone gave rise to around 10,000 DO11.10 cells per million lymph node cells. Interestingly only 5 ng p7.OVA per animal was sufficient to cause this proportion to more than double in size. However further dose increases did not result in corresponding population increases until a dose of 0.1 μg/mouse. At and above this dose, the DO11.10 population in the draining lymph nodes was significantly increased to an average of around 50,000 per million lymph node cells.

### 4.2.6. Dose-dependent proliferation is associated with a corresponding increase in cytokine potential.

Lymph node cells from the dose-response experiment above were cultured with or without restimulation with $OVA_{323-339}$ in ELISpot assays to detect cytokine production in response to restimulation (Figure 4-7). Several cytokines were tested: IL-2 as a general marker of T cell activation and, as seen in 0Figure 4-5, an indicator of proliferation; IL-4 and IFN-γ to determine a TH1/TH2 ratio and IL-10 because of its association with T-cell tolerance and relevance to later parts of this study.

Lymph node cells cultured in the absence of antigen were unable to generate any of the cytokines measured. Incubation with antigen, however, stimulated the production and secretion of all four. In each case the number of cytokine-producing cells was dependent on the immunisation dose of p7.OVA. IL-2 production followed the same pattern as the *in vivo* proliferation seen in 0Figure 4-3; a sigmoidal curve with maximal numbers at 0.1 μg/mouse p7.OVA and above. The number of IL-4 producers also followed this pattern although at roughly half the number of IL-2-producers. IFN-γ- and IL-10-producing cells were much less frequent and the increase in their numbers showed a more linear

relationship with p7.OVA dose. The ratio of IL-4-producing to IFN-γ-producing responders was roughly 5:1.

ELISpot is a very sensitive technique for detecting cytokine-producing cells; it can readily detect fewer than 100 cytokine-producing cells per million. A disadvantage to this technique, however, is that it doesn't easily allow for further phenotyping of cytokine-producing cells. To establish that the cytokine-producing cells detected in Figure 4-7 were indeed the adoptively transferred DO11.10 cells, the experiment was repeated using mice that either did or did not receive an adoptive transfer (Figure 4-8). As seen previously, 1000-2000 per million lymph node cells from immunised mice that received an adoptive transfer of DO11.10 cells secreted IL-2 and/or IL-4 whilst cells from unimmunised mice did not. In contrast, in mice that did not receive a DO11.10 adoptive transfer, these cytokines were not produced irrespective of immunisation. Thus the cytokine-producing cells seen in figure 4-7 were produced by adoptively transferred DO11.10 cells.

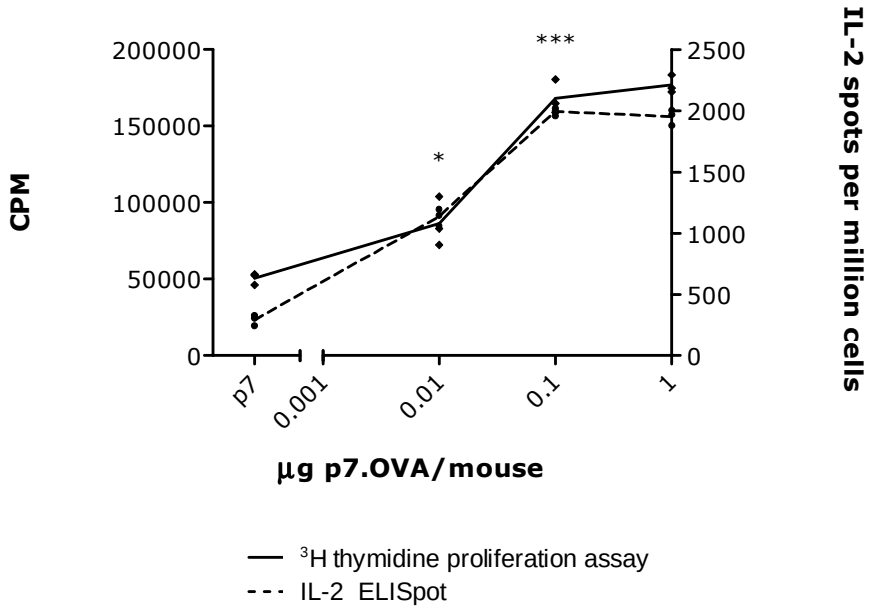



**Figure 4-5. p7.OVA dose-dependent increase in proliferation and IL-2 production following PMDD**

Following adoptive transfer of DO11.10 cells, recipient mice (3 per group) were immunised with 0.01, 0.1 or 1.0 µg p7.OVA. Control mice received empty p7 vector. At various points post-immunisation mice were sacrificed and inguinal lymph nodes harvested. Lymph node cells were restimulated with 1µM $OVA_{323-339}$ peptide in both thymidine-incorporation and IL-2-ELISpot assays. Each point (diamonds=thymidine incorporation; circles=ELISpot) represents an individual mouse.

*** $p<0.001$; * $p<0.05$ by one-way ANOVA as compared to p7 control

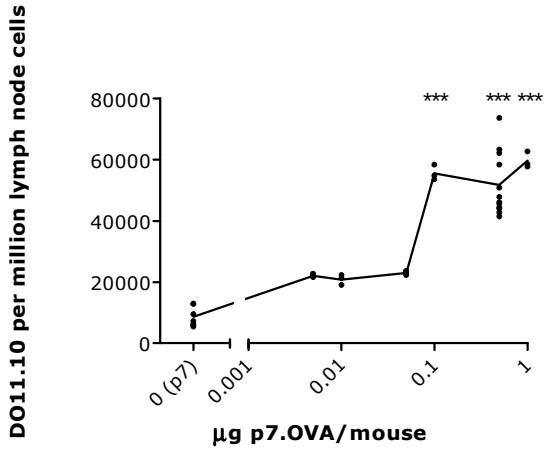



**Figure 4-6. The increase in lymph node DO11.10 cells is dependent on the dose of p7.OVA.**
Following adoptive transfer of DO11.10 cells, recipient mice (3 per group) were immunised with various doses of p7.OVA. Control mice received empty p7 vector. 72 h post-immunisation mice were sacrificed and inguinal lymph nodes harvested. Lymph node cells were stained with anti-CD4 and anti-KJ1.26. DO11.10 cells, identified as $CD4^+KJ1^+$, were enumerated by flow cytometry. Each point represents an independent experiment; the line represents the mean.
*** $p<0.001$ by one-way ANOVA as compared to p7 control

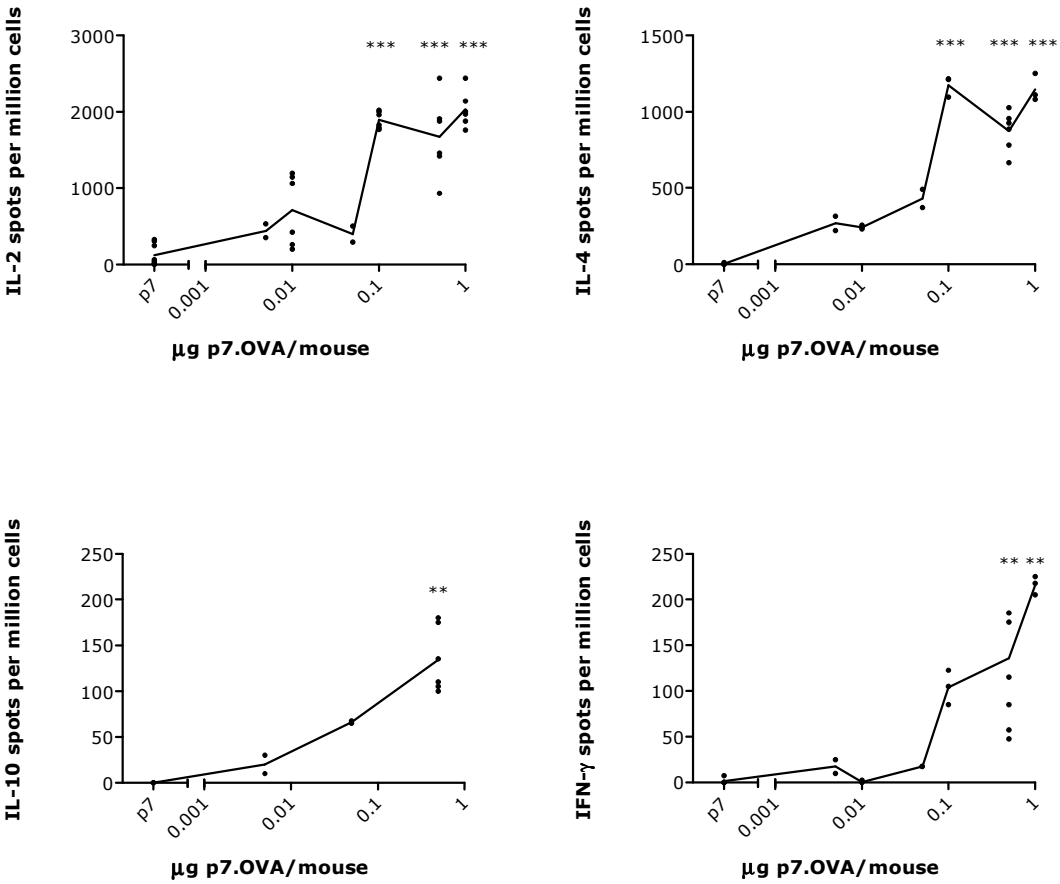



**Figure 4-7. Cytokine production in response to p7.OVA increases in a dose-dependent manner.**

Following adoptive transfer of DO11.10 cells, recipient mice (3 per group) were immunised with various doses of p7.OVA. Control mice received empty p7 vector. 72 h post-immunisation mice were sacrificed and inguinal lymph nodes harvested. Lymph node cells were restimulated with 1 µM $OVA_{323-339}$ peptide in cytokine ELISpot assays. Each point represents an independent experiment; the line represents the mean.

*** $p<0.001$; ** $p<0.01$ by one-way ANOVA as compared to p7 control

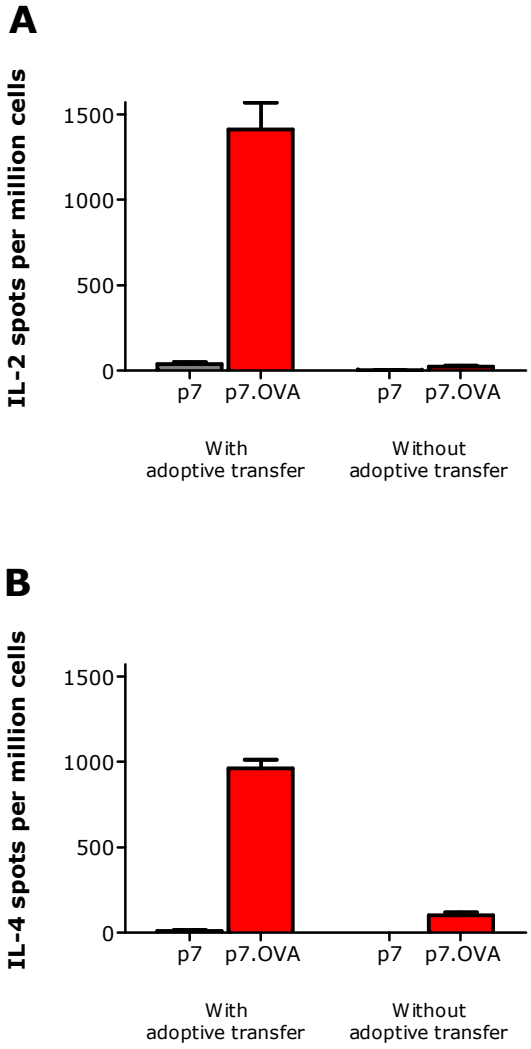



**Figure 4-8. Cytokine production requires adoptive transfer of DO11.10 cells.**

Balb/c mice (6 per group) either did or did not receive $1x10^6$ adoptively transferred DO11.10 cells. 1 day later, three mice were immunised with p7.OVA and three with empty p7 vector. 72 h post-immunisation mice were sacrificed and inguinal lymph nodes harvested. Lymph node cells were restimulated with 1μM $OVA_{323-339}$ peptide in IL-2 and IL-4 ELISpot assays (A and B, respectively). Data are the mean of triplicate wells of a single experiment.

### 4.2.7. Phenotypic changes in DO11.10 cells following PMDD

Following gene gun immunisation, harvested lymph node cells were triple-stained in order to examine cell surface phenotypic changes by flow cytometry. Upregulation of two common activation markers by $CD4^+KJ1.26^+$ cells was detected, accompanying their post-immunisation proliferation.

CD69 (an early marker of T cell activation) was upregulated on DO11.10 cells within 72 h of PMDD (Figure 4-9). This upregulation was not seen in $CD4^+KJ1^-$ cells. Immunisation with p7.OVA caused upregulation of CD69 on almost all DO11.10 cells with almost 70% expressing high levels after 72 h. After 96 h this had reduced to 50%. Interestingly, CD69 was also upregulated on $CD4^+KJ1.26^+$ cells in p7-immunised control mice. However the pattern of expression was different in this case: A distinct population of $CD69^+$ DO11.10 cells comprising around 50% of total $CD4^+KJ1.26^+$ cells was seen. This population remained roughly the same size until at least 96 h post-PMDD. It is interesting that this, presumably antigen-nonspecific, effect was specific to $CD4^+KJ1.26^+$ cells. This could be explained by a low level of pre-activation caused by the adoptive transfer: Although we have seen that division of the DO11.10 cells does not occur, a degree of nonspecific activation may be caused by agitation during the adoptive transfer process. In this case it would be logical, rather than to compare between $CD4^+KJ1.26^+$ and $CD4^+KJ1.26^-$ cells, to take $CD4^+KJ1.26^+$ cells from p7-immunised mice as the negative control. Comparison between these two groups shows that 60% of previously $CD69^-$ $CD4^+KJ1.26^+$ cells express this molecule at a high level within 72 h of immunisation with p7.OVA. Expression returns to control levels within the following 24 h.

Unlike CD69, control group expression of CD25 was not higher in $CD4^+KJ1.26^+$ cells than in $CD4^+KJ1.26^-$ cells: Figure 4-10 shows that 7-9% of all $CD4^+$ cells in empty p7-immunised mice express CD25. The same proportion was detected on $CD4^+KJ1.26^-$ cells in mice immunised with p7.OVA. However expression on $CD4^+KJ1.26^+$ cells increased to 22% 72 h after immunisation with antigen. Within a further 24 h expression had returned to control levels.

Both CD69 and CD25 are considered early activation markers. A limit to the data here is that it begins 72 h post-immunisation; before this time the low number of $CD4^+KJ1.26^+$ cells in the draining lymph node made analysis of cell surface markers on this subgroup impractical. Nevertheless, this data contributes to the general understanding of the model.

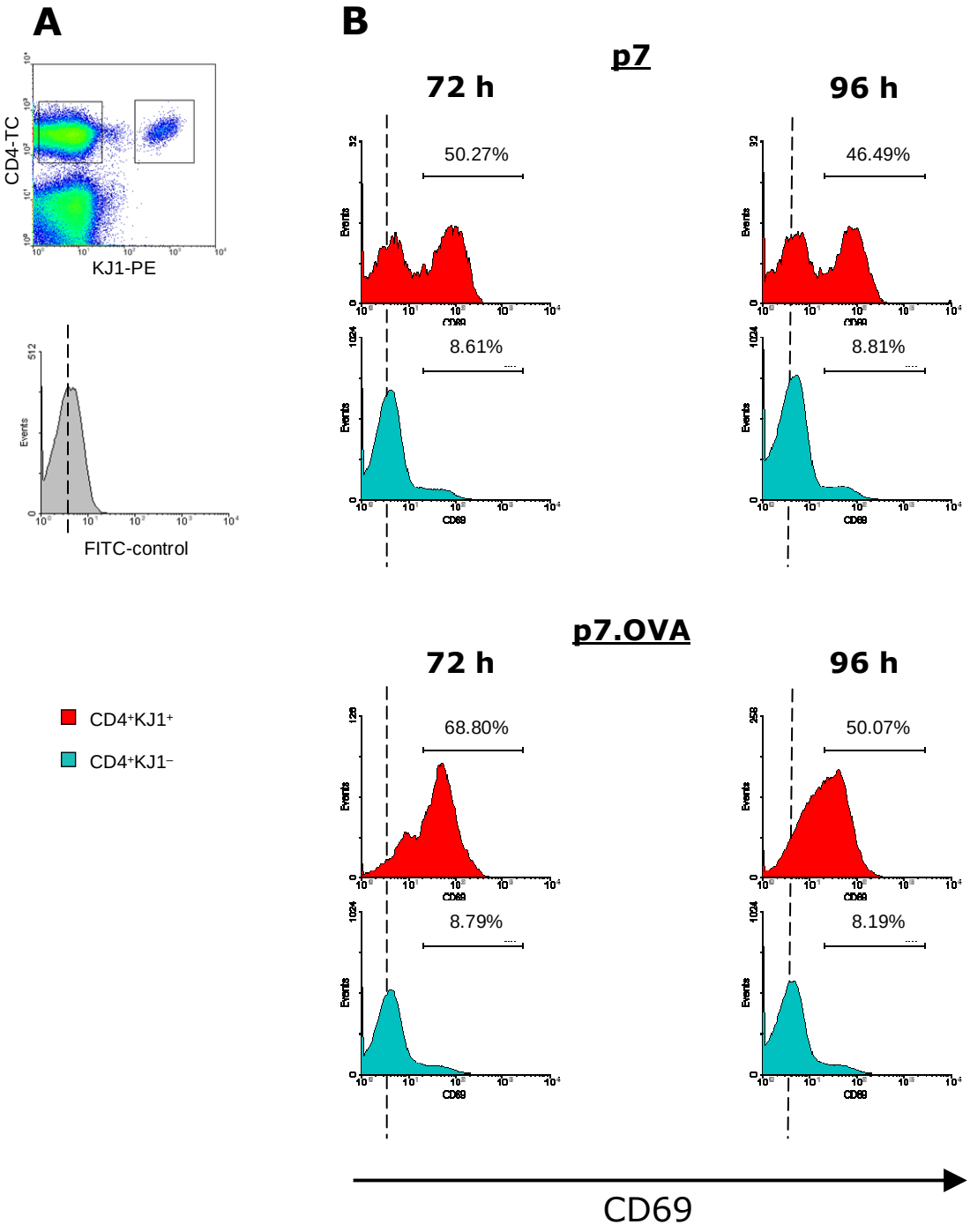



**Figure 4-9. Adoptively transferred DO11.10 cells upregulate CD69 after gene-gun immunisation with p7.OVA.**
$1x10^6$ DO11.10 cells were adoptively transferred into Balb/c recipients. 24 hours later mice were immunised with 1 µg p7 or 1 µg p7.OVA. 72 or 96 h post-immunisation inguinal lymph nodes were harvested and lymphocytes triple-stained with anti-CD4-TC, anti-KJ1.26-PE and anti-CD69-FITC. $CD4^+KJ1^+$ and $CD4^+KJ1^-$ lymphocyte populations were gated (A) and CD69 expression of these two populations examined (B).

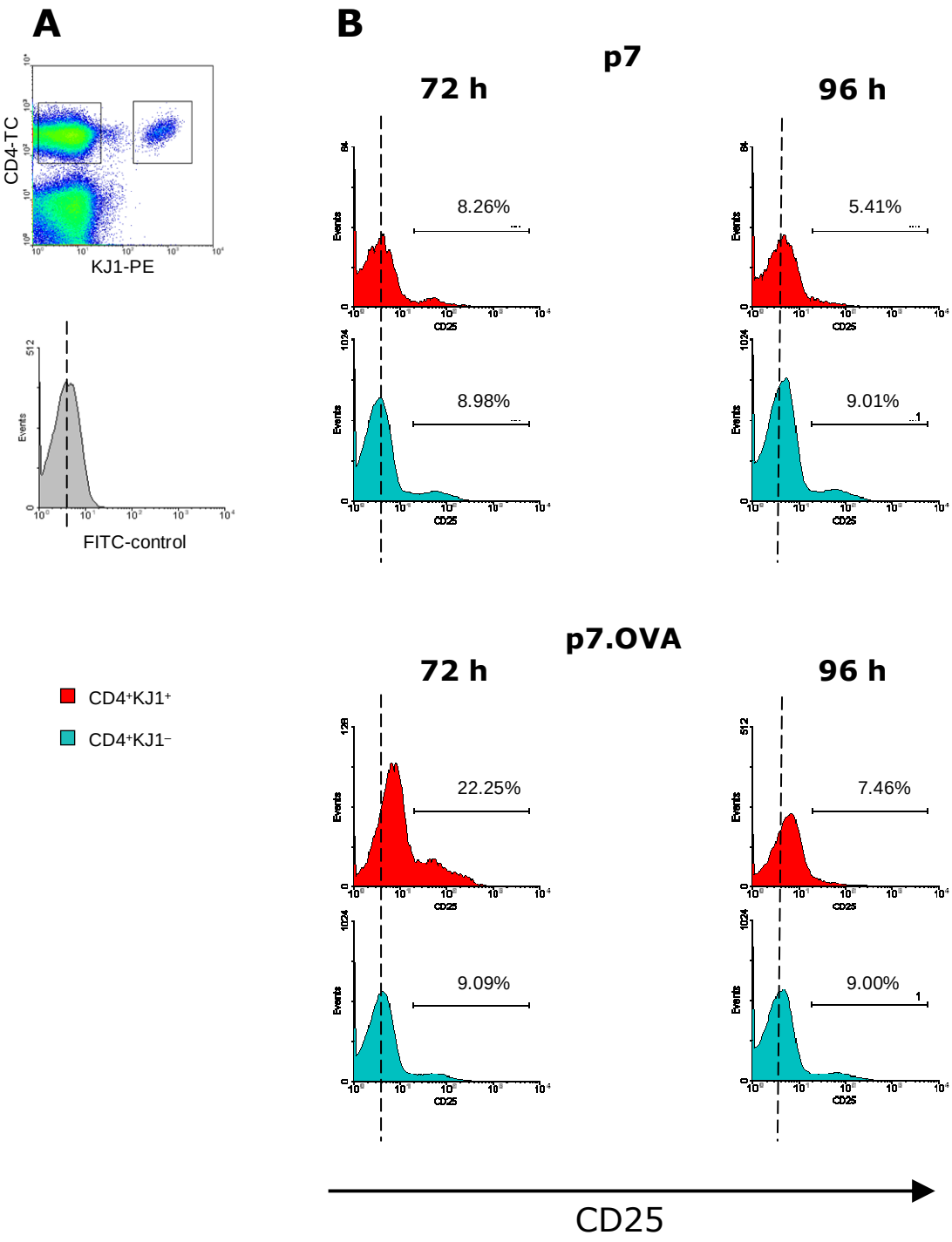



**Figure 4-10. Adoptively transferred DO11.10 cells briefly upregulate CD25 after gene-gun immunisation with p7.OVA.**
1x10$^6$ DO11.10 cells were adoptively transferred into Balb/c recipients. 24 hours later mice were immunised with 1 µg p7 or 1 µg p7.OVA. 72 or 96 h post-immunisation inguinal lymph nodes were harvested and lymphocytes triple-stained with anti-CD4-TC, anti-KJ1.26-PE and anti-CD25-FITC. $CD4^+KJ1^+$ and $CD4^+KJ1^-$ lymphocyte populations were gated (A) and CD25 expression of these two populations examined (B).

## 4.3. Discussion

The adoptive transfer of TCR-transgenic DO11.10 T cells into Balb/c recipients allowed the study of the $CD4^+$ T cell response to PMDD-administered antigen. Both the magnitude and kinetics of the response have been examined, as well as the cytokine potential of the resulting antigen-experienced T cells. Although Creusot *et al*. have previously used a similar model, both the plasmid vector and the mouse strain used are different in this case. In order to facilitate the later study of the effect of IL-10 on the responding T cells, it was first necessary to characterise the nature of this specific model which employs the p7 vector and the $H\text{-}2^d$ Balb/c mouse.

### 4.3.1. The suitability of the DO11.10 adoptive transfer model for the examination of the effect of IL-10 on the $CD4^+$ T cell response to PMDD immunisation

TCR-transgenic DO11.10 cells can be clearly identified using the KJ1.26 clonotypic monoclonal antibody. Their quantification amongst total splenocytes allows the adoptive transfer of a specific fixed number simply by adjusting the total number of splenocytes transferred. $1x10^6$ DO11.10 cells allowed to equilibriate within the recipient routinely results in a lymph node population of 0.5%-1.0%. This number is high enough to allow clear identification and examination of the population. However, unlike the population within the original DO11.10 host, the population is not so dominant so as to preclude the interpretation of any results as a description of the natural response.

The *in vivo* proliferative response to PMDD antigen cen be detected 3 d post-immunisation. Peak numbers, however, occur on day 5; two days later than similar experiments that used protein antigen(Rogers *et al.*, 1997). This delay could be attributable to the need for *in situ* synthesis from the cDNA template and subsequent accumulation of processed antigen.

These kinetics prohibit more detailed examination of the DO11.10 population by flow cytometry at any time earlier than 72 h post-immunisation as the number of gated events is simply too small for any meaningful analysis of surface phenotype or intracellular cytokine staining. ELISpot, as mentioned earlier, is more suited to the examination of smaller fractions of cytokine-producing cells.

### 4.3.2. Response to restimulation

Direct *ex vivo* examination of DO11.10 cells following a single PMDD immunisation can only characterise their response to that particular challenge. However later restimulation analyses can provide an insight into the lasting effect of the immunisation on their state of activation. The secretion of detectable levels of IL-2 (as well as other cytokines) by DO11.10 cells within 19 h of contact with $OVA_{323\text{-}339}$ demonstrates previous antigen experience and the effector state of the T cell. This provides a useful tool for the study of the effect of IL-10 on PMDD immunisation.

The cytokine ELISpots in this chapter demonstrate that the immune response to OVA in this model is $T_H2$-biased, with five times as many IL-4-producing as IFN-γ-producing responders. Interestingly, the number of IL-10-producing responders also increased with the dose of p7.OVA, the population being of similar size to that of the IFN-γ-producers.

### 4.3.3. Dose response

PMDD is different from traditional immunisation regimens because antigen (or at least the gene encoding antigen) is directly deposited intracellularly. The data therefore reflect responses to an increased number of plasmid copies per bead (and therefore per cell); not to increased numbers of transfected cells. Expression and antigen presentation may not be directly proportional to the copy number of antigen gene. It is therefore especially important to understand how the dose of antigen gene affects the response to antigen. In fact, a dose-dependent relationship was observed.

The gene gun delivers DNA indiscriminately at the site of administration. Although dendritic cells are directly transfected[(Larregina *et al.*, 2001; Morita *et al.*, 2001)], non-APC-specialised cells such as keratinocytes are, purely through their numbers, more frequently transfected[(Lu *et al.*, 1996)]. Although they could process and present antigen themselves via the MHC class I pathway, it is more likely that the main role of these cells in the generation of the immune response is that of synthesis and secretion of antigen for uptake and cross-presentation by professional APC[(Dean *et al.*, 2003; Stoitzner *et al.*, 2006)].

One or both of two general mechanisms may be responsible for the dose-dependent increase in proliferation and activation of DO11.10 cells.

1) Antigen remains intracellular: The increased copy number per cell results in increased synthesis of antigen. A larger antigen dose can produce a stronger activation signal and thus increased *quality* of presentation to $CD4^+$ T cells[(Ruedl *et al.*, 2000; Rothoeft *et al.*, 2006)].

2) Antigen is secreted: The higher concentration of antigen resulting from increased synthesis allows its diffusion over a larger area and thus its uptake and presentation by a larger number of APC i.e. increased *quantity* of antigen presentation to $CD4^+$ T cells.

Whether the synthesised OVA protein was retained intracellularly or secreted was not directly tested. However the wild-type 48 amino acid secretory signal was encoded intact in the OVA transgene so a working assumption would be that secretion did occur. A cytoplasmic version of the gene lacking this sequence can be used to ensure retention[(Rowe *et al.*, 2006)]. This not being the focus of this study, the mechanism was not examined further. The relevant point for this and later discussion is that, unless specified, when the term "increased dose" is used, it refers to an increased intracellular dose of gene.

For later studies of the effect of p7.IL10 on the immune response to p7.OVA, it was important to ensure any measured differences were not a result of plasmid interference inhibiting expression of the antigen-containing vector. Although Figure 3-2 and Figure 3-3 demonstrate that coadministration of p7.OVA and p7.IL10 was possible, there remains the possibility that very minor inhibition occurs. Therefore, a minimum p7.OVA dose of 0.5 μg/mouse was established. This is five times higher than the lowest dose that resulted in peak proliferation and cytokine production and, therefore, any minor decrease in expression should not affect their amplitude. The maximum p7.OVA dose was prescribed by the capacity of the gene gun cartridges. The maximum reliable loading rate allows 0.5 μg DNA per cartridge and thus no more than 1 μg total DNA could be administered per mouse. However, to allow for the inclusion of other plasmids within the same cartridge, a maximum of 0.5 μg/mouse was loaded. 0.5 μg/mouse was used as the standard dose for p7.OVA in all later studies unless stated otherwise.

### 4.3.4. Gene gun as an adjuvant

A large increase in the number of $CD4^+KJ1.26^+$ cells in draining lymph nodes, lasting at least 120 h, was seen following immunisation with p7.OVA. In contrast, a small, transient increase was seen when mice were treated with empty p7 vector only. Nevertheless, this increase occurred in the absence of any exogenous antigen. Further examination showed that the transient increase in lymph node T cells was not caused by proliferation. It is therefore likely to be caused by a change in the rate of immigration to and/or emigration from the lymph node.

The increase in $CD4^+$ T cells in the lymph node in the absence of antigen suggests that gene gun is immunostimulatory – its own adjuvant. Indeed, no external adjuvant is often required for PMDD immunisation, just the antigen-encoding plasmid. Two factors that may contribute to this phenomenon are the plasmid DNA itself and the cell damage caused by PMDD.

Plasmid DNA (and bacterial DNA in general) has been known for some time to be immunostimulatory (reviewed in (Yamamoto *et al.*, 2000)). The induced maturation of several cell types, including dendritic cells, has been attributed to unmethylated CpG sequences(Yamamoto *et al.*, 1992a; Krieg *et al.*, 1995; Segal *et al.*, 1997; Sparwasser *et al.*, 1997; Jakob *et al.*, 1998) whose presence is suppressed in mammalian DNA(Schorderet and Gartler, 1992). More recently the receptor for these sequences was identified as toll-like receptor 9 (TLR-9)(Hemmi *et al.*, 2000). CpG sequences, either as exogenous adjuvant with protein antigen(Chu *et al.*, 1997; Lipford *et al.*, 1997; Weiner *et al.*, 1997) or as an endogenous part of DNA vaccine(Sato *et al.*, 1996; Klinman *et al.*, 1997), have been shown to enhance immunogenicity and to modulate the response towards a $T_H1$ bias. In addition, CpG sequences have been shown to induce the migration of Langerhans cells(Ban *et al.*, 2000).

Disruption of cell integrity caused by gene gun bombardment may also activate antigen presenting cells and induce their migration. Necrotic cells, in particular the heat shock proteins which they release, can activate dendritic cells via toll-like receptor- (TLR-) 2 and/or 4 and the NFκB pathway(Gallucci *et al.*, 1999; Basu *et al.*, 2000; Sauter *et al.*, 2000; Vabulas *et al.*, 2002).

Thus both plasmid DNA and the cell damage caused by the gene gun may account for the transient increase in $CD4^+KJ1.26^+$ cells found in the draining lymph node following administration of empty p7 vector. The mechamism by which these immune stimuli might affect the rate of T cell immigration and/or emigration remains unknown but could result from direct or indirect changes in T cell expression of CD69 and S1P1 which are known to regulate migration through lymph nodes(Matloubian *et al.*, 2004; Shiow *et al.*, 2006). In addition, an increased number of DC in the lymph nodes could increase the time which T cells spend sampling in the lymph node.

### 4.3.5. Conclusion

The DO11.10 adoptive transfer model has previously been used to examine the immune response to OVA antigen. This chapter demonstrates that it is suitable for the study of gene gun-administered immunisation strategies. This model will be used to establish whether the coadministration of the IL-10-encoding plasmid p7.IL10 is effective at inhibiting the immune response to p7.OVA and/or inducing a tolerant state in recipient animals.

# 5. Effect of IL-10 on the response to antigen

## 5.1. Introduction

As mentioned earlier, one of the many advantages to genetic immunisation is the opportunity to choose from a range of immunomodulatory genes or gene products to administer alongside the antigen-encoding gene. This was first shown using intramuscular injection of genes encoding both antigen and cytokine[(Xiang and Ertl, 1995)] and was later followed by injection of various recombinant cytokines into the site of gene-gun vaccination[(Irvine *et al.*, 1996)]. The next advance was gene-gun vaccination of a fusion of *ovalbumin* and *il-12* genes[(Maecker *et al.*, 1997)] which was later shown to be a more effective vaccine than the recombinant cytokine method of Irvine et al.[(Kim *et al.*, 1997c)].

Rodent models of immune disease lend themselves to the study of immunomodulation and have been an important part in the study of IL-10 in this capacity. Specifically relevant to this work is the utilisation of the IL-10 gene, *il-10*, to provide this cytokine.

Early work showed that intramuscular injection of an IL-10-expressing plasmid was able to significantly reduce the incidence of diabetes in NOD mice when administered prophylactically[(Nitta *et al.*, 1998)]. One disadvantage to this technique is the requirement for repeated administration; another is the quantity of plasmid vector required. Viral vectors containing *il-10* address both these limitations. Adenovirus vectors have been shown to prevent induced colitis[(Cua *et al.*, 1999; Barbara *et al.*, 2000)] but only in the case of colitis due to IL-10 deficiency have they also proven to be therapeutic[(Lindsay *et al.*, 2001; Lindsay *et al.*, 2002; Lindsay *et al.*, 2003)]. Similarly, protection of Nonobese Diabetic (NOD) mice from developing diabetes required treatment at an early age, before the onset of hyperglycemia. Adeno-associated-virus encoding vIL-10 protected NOD mice from becoming hyperglycemic if administered before 6 weeks of age[(Yang *et al.*, 2002)].

More success was seen in rodent models of arthritis (collagen-induced arthritis; CIA). Systemic administration of adenovirus vector encoding the Epstein-Barr Virus IL-10 homologue (vIL-10) at the onset of symptoms prevented the development of disease in murine collagen-induced arthritis[(Apparailly *et al.*, 1998)]. However, a later study suggested that this effect was only possible using viral doses high enough to cause hepatoxicity[(Whalen *et al.*, 1999)]. This study also showed that local administration of the vector into the periarticular area of mouse paws was effective in preventing development of CIA. Surprisingly, injection into one paw proved to be sufficient to prevent arthritis in the remaining paws. In addition, human IL-10-encoding adenoviral vector injected into the knee joint has been shown to suppress the onset of arthritis in the ipsilateral paw as well as in the knee itself. Interestingly, reduced liver inflammation compared to that caused by empty adenovirus suggests that hepatoxicity caused by the viral vector, as mentioned above, may be partially suppressed by the IL-10 it itself encodes[(Quattrocchi *et al.*, 2001)]. A study comparing IV and local administration of such a vector agrees that the latter is more effective in preventing disease but that neither is able to reverse established arthritis[(Ma *et al.*, 1998)]. Similar results were obtained in a rabbit model of arthritis[(Lechman *et al.*, 1999; Keravala *et al.*, 2006)]. In this case, the

inducing antigen was entirely foreign and it was possible to show that the therapeutic effect was antigen-specific[(Lechman *et al.*, 2003)].

Retroviral vectors have also been used as IL-10 vectors but, in this case, indirectly via pre-transfection of DC. Whereas, *in vitro*, the immunostimulatory ability of these cells was diminished[(Takayama *et al.*, 1998; Takayama *et al.*, 1999)], attempts to utilise them *in vivo* resulted in limited success[(Takayama *et al.*, 2001b; Moore *et al.*, 2004)]. Injection of such vectors directly into cardiac allografts has been shown, however, to extend the period of graft acceptance [(Qin *et al.*, 1996; Qin *et al.*, 2001)].

Safety concerns over the use of viral vectors and the established importance of the intestinal flora promote the concept of local treatment of colitis with IL-10-secreting bacteria. *Lactococcus lactis*, transformed with an IL-10-encoding plasmid, was shown to alleviate inflammation in two murine models of colitis[(Steidler *et al.*, 2000)]. Interestingly, the bacterium displays an affinity towards inflamed tissue[(Waeytens *et al.*, 2007)]. Refinement of the genetic modification[(Steidler *et al.*, 2003)] and improvement of delivery[(Huyghebaert *et al.*, 2005b; Termont *et al.*, 2006)] have allowed this treatment to reach Phase I clinical trials[(Braat *et al.*, 2006)].

Local administration of IL-10 or its gene allow therapy to be targeted to specific tissues and occasionally, due to the confined nature of some immune disorders, this may be sufficient. However, cytokine secreted into the general environment has the potential to exert its effects on every antigen presentation event in the vicinity. The immunological synapse allows cytokines to be directed more specifically toward antigen-specific T cells that cluster around antigen-presenting DC[(Creusot *et al.*, 2002)]. This allows both the DC itself and previously activated and polarised T cells to bias the immune response to a presented antigen through the paracrine secretion of cytokines[(Creusot *et al.*, 2003a)].

Targeting the IL-10 gene to dendritic cells would exploit this existing mechanism for increasing antigen specificity. Gene gun technology allows the direct transduction of skin DC. The intention of this chapter is to examine whether gene gun administration of the IL-10 gene alongside that of antigen can inhibit the response to that antigen.

## 5.2. Results

### 5.2.1. Coadministration of p7.IL10 inhibits the response to antigen

$1x10^6$ DO11.10 cells were adoptively transferred into naïve Balb/c recipients. 24 h later mice were immunised with cartridges containing both 0.5 μg p7.OVA and either 0.5 μg, 0.17 μg or 0.05 μg p7.IL10 ("Relative quantity" 1, 0.3 and 0.1, respectively). One control group received 0.5 μg p7.OVA only ("Relative quantity" 0) and a second received empty p7 vector only ("p7"). 72 h post-immunisation draining lymph nodes were harvested and $CD4^+KJ1^+$ cells enumerated by flow cytometry (Figure 5-1). Consistent with earlier studies, whereas in mice immunised with empty vector DO11.10 cells never comprised more than 500 per million lymph node cells, in those that received p7.OVA this figure reached 4000 per million. Coadministration of p7.IL10 at a 1:10 ratio had no effect

on the number of DO11.10 cells in the lymph node. In contrast, coadministration at a 1:3 or 1:1 ratio reduced the number of DO11.10 cells by around 40%.

Cytokine expression by the same lymph node cells was analysed by ELISpot (Figure 5-2). IL-2 was expressed by roughly 1500 cells per million in mice immunised with p7.OVA only. This proportion did not change upon coadministration of p7.IL10 at a 1:10 ratio. However when the ratio was increased to 1:3 the proportion of lymph node cells expressing IL-2 decreased to around 500 per million. Further increasing the dose of p7.IL10 had no further inhibitory effect on IL-2 expression. Fewer cells produced IL-4, IL-10 and IFN-γ in response to restimulation with $OVA_{323-339}$: 1000, 300 and 150 per million lymph node cells, respectively. However the expression of these three cytokines was inhibited in a similar dose-dependent manner whereby p7.IL10 had no effect at a 1:10 ratio but at 1:3 and 1:1 ratios, the number of cytokine-producing cells was reduced by 50-70%.

CD62L (L-selectin) is expressed on naïve T cells, allowing entrance into secondary lymphoid tissue. Its expression is downregulated upon response to antigen, facilitating migration to the periphery. CD62L expression on $CD4^+$ T cells was examined by flow cytometry following the aforementioned dose-response protocol (Figure 5-3). In OVA-naïve mice (p7), 80% of both antigen-specific DO11.10 ($CD4^+KJ1.26^+$) and antigen-nonspecific ($CD4^+KJ1.26^-$) expressed CD62L. Whereas immunisation with p7.OVA had no effect on nonspecific T cells, it induced downregulation in a futher 30% of DO11.10 cells. This effect was slightly reduced by the coadministration of p7.IL10 at 1:3 and 1:1 ratios.

The lowest dose ratio of p7.IL10 to consistently, significantly inhibit the response to p7.OVA was 1:3 i.e. 0.17 μg p7.IL10 to 0.5 μg p7.OVA. For this reason, this dose was used in all later experiments.

### 5.2.2. Coadministration of p7.IL10 delays the proliferative response to antigen

72 h post-immunisation was chosen as suitable to measure the effect of p7.IL10 because initial experiments had shown this timepoint to provide near-maximal cell proliferation and cytokine production whilst still providing a snapshot of the early stages of the response. Nevertheless it was important to examine the temporal effect(s) of p7.IL10 on this initial response.

$1x10^6$ DO11.10 cells were adoptively transferred into naïve Balb/c recipients. 24 h later mice were immunised with cartridges containing either p7.OVA or a 1:3 ratio of the two plasmids as described in 5.2.1. Earlier experiments had shown the complete unresponsiveness of DO11.10 cells to empty p7 plasmid. It was therefore decided that, in this experiment, this control was unnecessary. 24, 72 or 120 h post-immunisation draining lymph nodes were harvested and cytokine production by lymph node cells analysed by ELISpot (Figure 5-4). At 24 h there was no difference in expression in any of the cytokines measured between groups: Expression of IL-4, IFN-γ and IL-10 was undetected and only 400 per million lymph node cells expressed IL-2. After 72 h the proportion which expressed IL-2 had

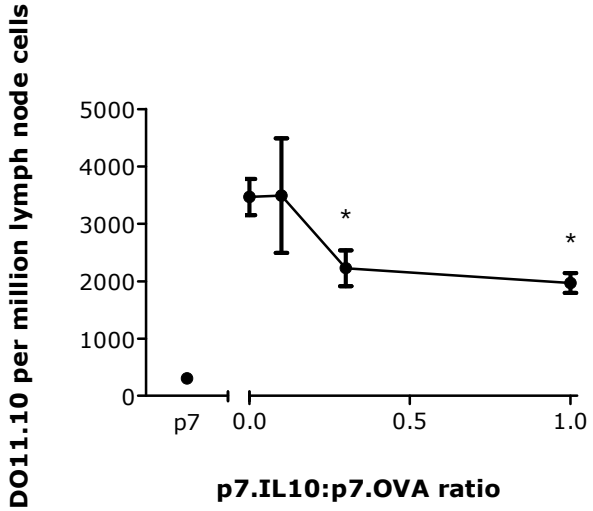



**Figure 5-1. p7.IL10 inhibits the proliferative response to p7.OVA in a dose-dependent manner.**
Following adoptive transfer, mice (3 per group) were immunised with 0.5 μg p7.OVA alone, or with 0.05 μg, 0.17 μg or 0.5 μg p7.IL10. Control mice received empty p7 only. 72 h post-immunisation lymph nodes were harvested and DO11.10 cells counted by flow cytometry. Data are the mean of two independent experiments.
* $p<0.05$ by one-way ANOVA as compared to p7.OVA ("Relative quantity 0.0") group

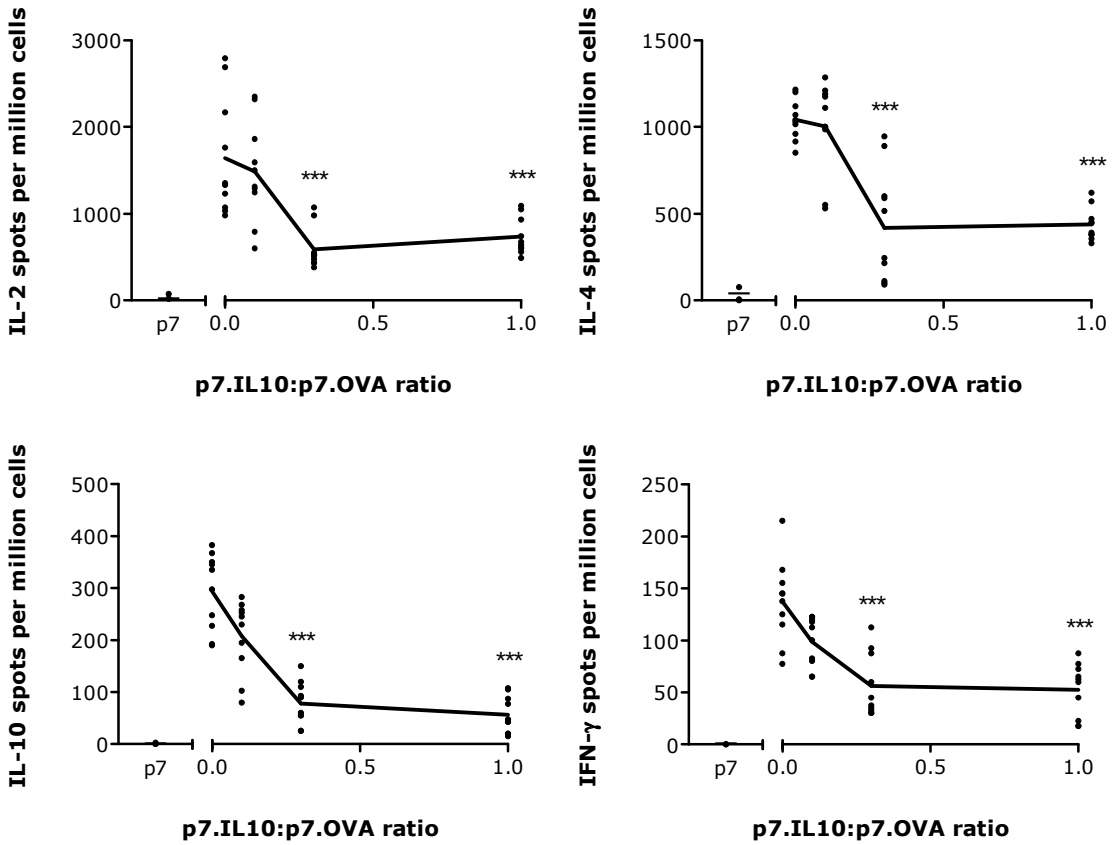



**Figure 5-2. p7.IL10 inhibits the cytokine response to p7.OVA in a dose-dependent manner.**
Following adoptive transfer, mice (3 per group) were immunised with 0.5 μg p7.OVA alone, or with 0.05 μg, 0.17 μg or 0.5 μg p7.IL10. Control mice received empty p7 only. 72 h post-immunisation lymph nodes were harvested and cytokine expression examined by ELISpot. Data are the mean of four independent experiments.
*** $p<0.001$ by one-way ANOVA as compared to p7.OVA ("Relative quantity 0.0") group

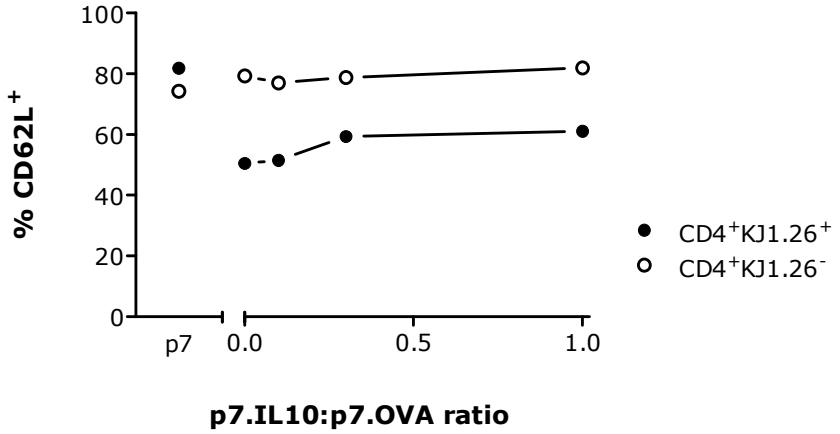



**Figure 5-3. p7.IL10 inhibits the downregulation of CD62L in response to p7.OVA.**
Following adoptive transfer, mice (3 per group) were immunised with 0.5 μg p7.OVA alone, or with 0.05 μg, 0.17 μg or 0.5 μg p7.IL10. Control mice received empty p7 only. 72 h post-immunisation lymph nodes were harvested and CD62L expression on $CD4^+KJ1.26^+$ cells examined by flow cytometry. Data are representative of two independent experiments.

increased to over 1000 per million in mice that had received p7.OVA alone whereas in those that had received coadministered p7.IL10 it remained around 400 per million. During the next 48 h the proportion of IL-2-producers in the p7.OVA group remained at around 1000 per million whilst those in the group that received coadministered p7.IL10 increased to a similar proportion. Although the proportion of IL-4-producing cells from p7.OVA recipients continued to increase through all timepoints measured (to a maximum of 800 per million), a similar pattern was observed: In lymph nodes from mice that received coadministered p7.IL10 no increase in IL-4 expression was observed before 72 h but by 120 h the number of IL-4-producers had increased rapidly to a similar proportion as that seen in the p7.OVA group. A p7.IL10-induced delayed increase was also observed in the generation of IFN-γ- and IL-10-producing cells although both the absolute numbers and the differences between groups were much smaller for these cytokines.

To examine this delayed response to antigen in more detail, DO11.10 cells were stained with 5μM CFSE prior to adoptive transfer. As above, recipient mice were immunised with p7.OVA alone or along with p7.IL10. Recipient Balb/c were culled and draining lymph nodes harvested at various timepoints post-immunisation. Division of DO11.10 cells was measured by flow cytometry (Figure 5-5). In both groups the number of $CD4^+KJ1^+$ cells present in the lymph node after 24 h was insufficient to examine CFSE staining. After 48 h there was a sufficient number but cell division had not occurred in either group. In mice which received p7.OVA only, proliferation of $CD4^+KJ1^+$ cells became evident after 60 h and accelerated until 84 h at which time over 1600 divisions had occurred. Over the next 12 h the rate of proliferation decreased, with a total of 2000 divisions by 96 h post-immunisation. The proliferative response was delayed in mice which were coadministered p7.OVA and p7.IL10: From 60 h to 84 h the number of $CD4^+KJ1^+$ cell divisions remained roughly 40% of the number of divisions in mice which received p7.OVA only. However between hours 84 and 96, while the proliferation rate in mice which received p7.OVA only decreased, in mice which received both plasmids proliferation accelerated: By 96 h a total of 1700 $CD4^+KJ1^+$ divisions had occurred; only 15% less than in mice that received p7.OVA alone.

### 5.2.3. The inhibitory effect of p7.IL10 on the response to p7.OVA is exaggerated by a repeat dose

Several tolerising regimes involve repeated or continuous exposure to antigen under noninflammatory conditions(Larche *et al.*, 2006). To examine the effect of a repeat dose in this model, Balb/c recipients which had received adoptive transfer and immunisation as described above were allowed to rest. 28 days later mice received a second dose of the same immunisation (either empty p7, p7.OVA or p7.OVA + p7.IL10). In order to identify longer-term differences between groups, rather than merely the delay described in 5.2.2, lymph nodes were harvested 5 days after this second immunisation.

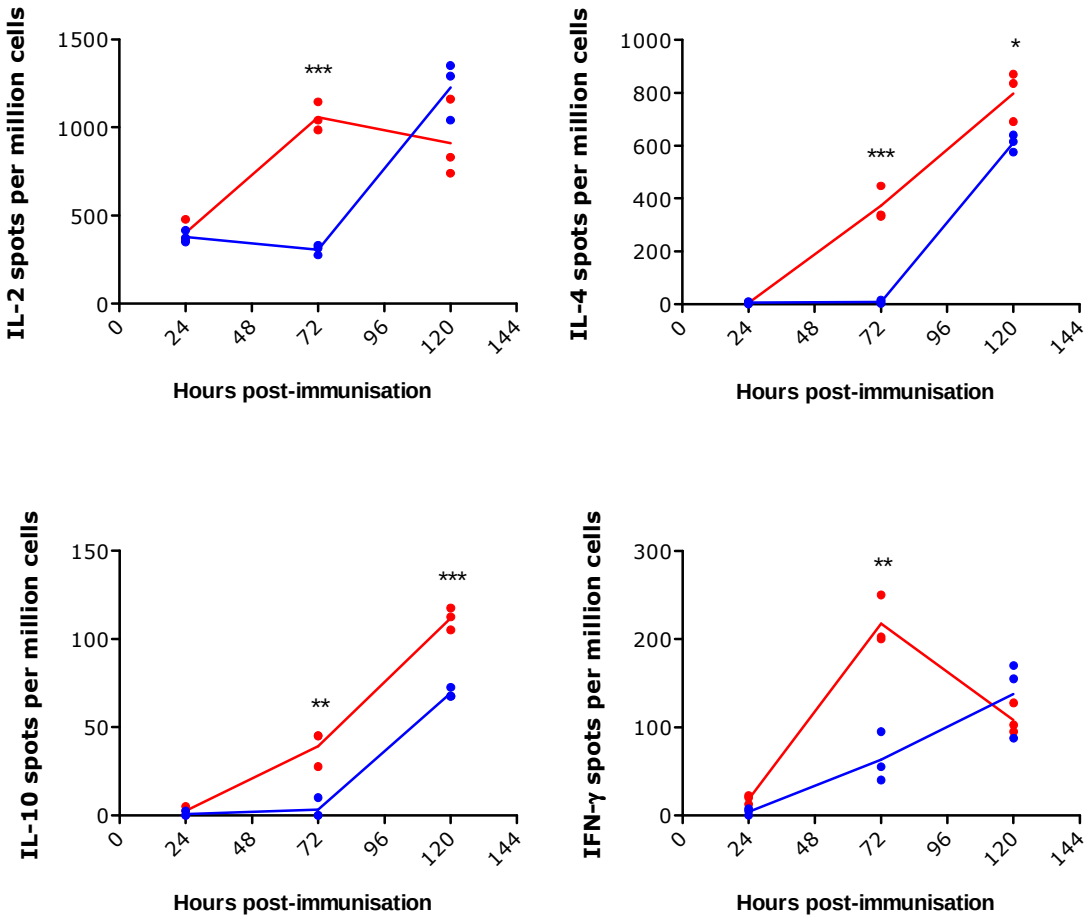



**Figure 5-4. p7.IL10 delays the cytokine response to p7.OVA.**
Following adoptive transfer, mice (3 per group) were immunised with 0.5µg p7.OVA alone (red lines) or together with 0.17 µg p7.IL10 (blue lines). 24, 72 and 120 h post-immunisation draining lymph nodes were harvested and cytokine expression examined by ELISpot. Data are the mean of three independent experiments.
*** $p<0.001$; ** $p<0.01$; * $p<0.05$ by t-test between groups at each timepoint

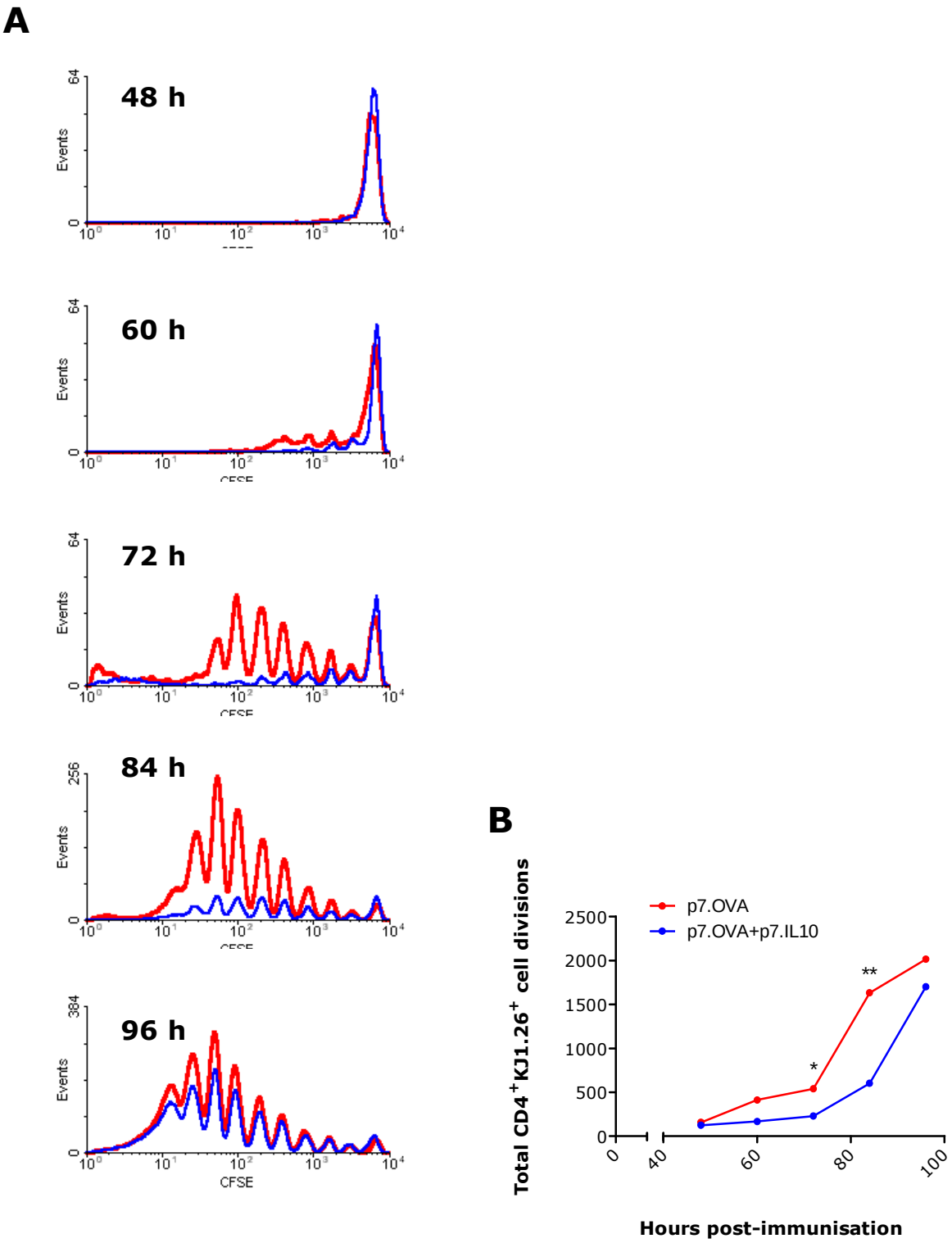



**Figure 5-5. p7.IL10 delays the proliferative response to p7.OVA.** Prior to adoptive transfer, splenocytes were stained with 5 μM CFSE. Following adoptive transfer, mice were immunised with 0.5 μg p7.OVA alone, or together with 0.17 μg p7.IL10. Control mice received empty p7 only. 72 h post-immunisation lymph nodes were harvested and CFSE-fluorescence of DO11.10 cells examined by flow cytometry (A). Total cell divisions were calculated as described in Materials and Methods (B). Data are means of 3 mice per group. Graph is representative of two independent experiments. ** $p<0.01$; * $p<0.05$ by t-test between groups at each timepoint

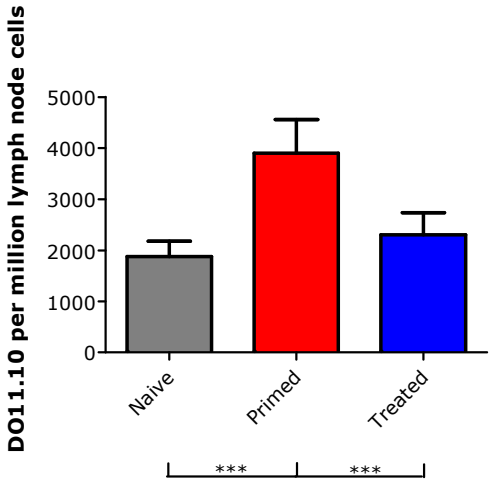



**Figure 5-6. The inhibitory effect of p7.IL10 on the proliferative response is exaggerated after a repeat dose.**
Mice (3 per group) received two doses, separated by 4 weeks, of empty p7 ("naive"), p7.OVA ("primed") or p7.OVA+p7.IL10 ("treated"). 5 days after the second immunisation inguinal lymph nodes were harvested and DO11.10 cells counted by flow cytometry. Data are the mean of four independent experiments.
*** $p<0.001$ by one-way ANOVA

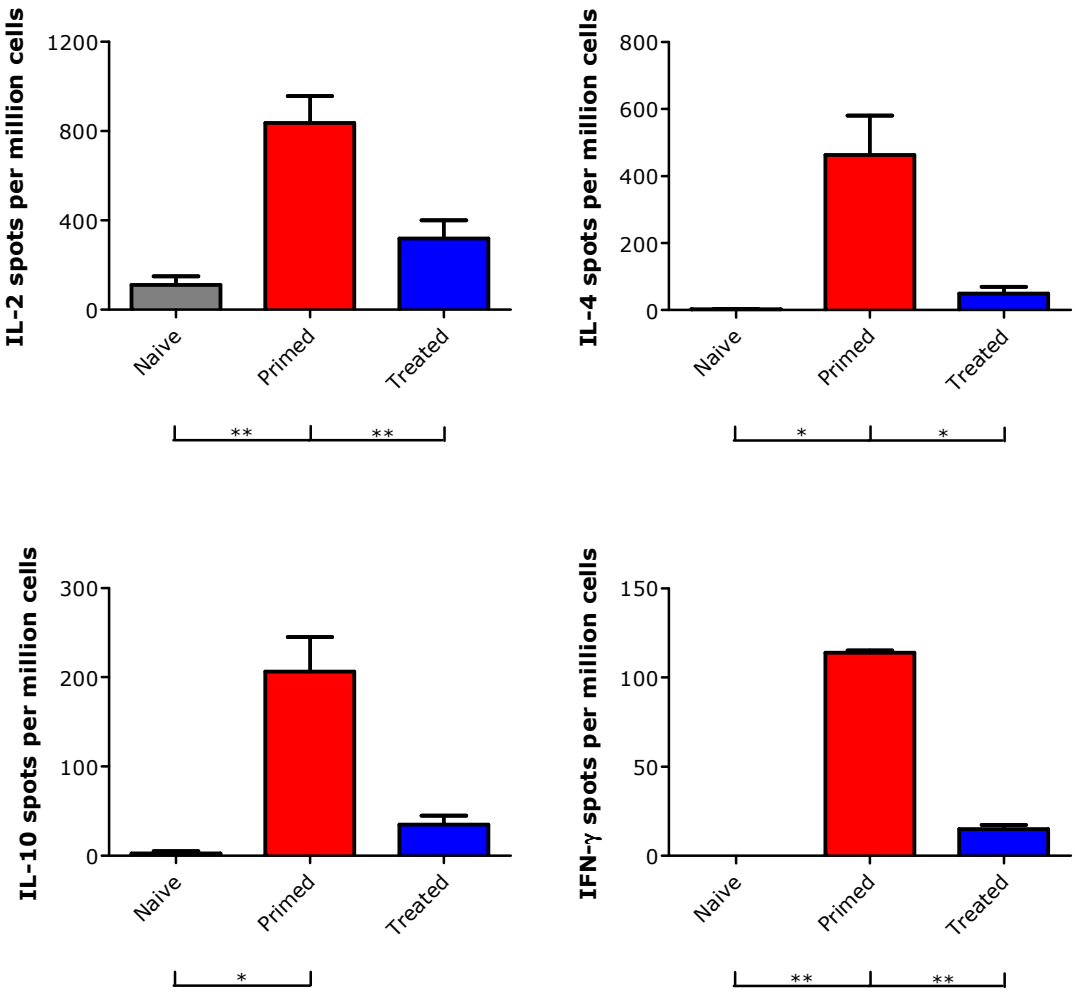



**Figure 5-7. The inhibitory effect of p7.IL10 on the cytokine response is exaggerated after a repeat dose.**
Mice (3 per group) received two doses, separated by 4 weeks, of empty p7 ("naive"), p7.OVA ("primed") or p7.OVA+p7.IL10 ("treated"). 5 days after the second immunisation inguinal lymph nodes were harvested and the cytokine response analysed by ELISpot. Data are the mean of two independent experiments.
** $p<0.01$; * $p<0.05$ by one-way ANOVA

Following immunisation with two doses of p7.OVA, lymph nodes contained roughly 4000 DO11.10 per million cells (Figure 5-6). Coadministration of p7.IL10 halved this number. Interestingly, double immunisation with empty p7 vector resulted in nearly 2000 DO11.10 per million lymph node cells, too. Therefore, under this protocol, coadministration of p7.IL10 reduced the number of DO11.10 cells in the lymph node to background levels.

Similar to earlier experiments, in mice that received p7.OVA alone, IL-2, IL-4, IFN-γ and IL-10 were produced by roughly 1000, 600, 100 and 200 per million lymph node cells, respectively. In the previous experiments coadministration of p7.IL10 (at a 1:3 ratio) resulted in a 15-20% reduction of IL-2 producers (Figure 5-2). However, upon receiving a repeat dose the inhibition was more exaggerated: 60% fewer DO11.10 cells in the draining lymph nodes of recipient mice expressed IL-2 when p7.IL10 was coadministered compared to immunisation of p7.OVA alone (Figure 5-7). The number of IL-4-producing DO11.10 cells was reduced by 80% and the numbers of IFN-γ- and IL-10-producers also decreased by similar proportions.

### 5.2.4. The inhibitory effect of p7.IL10 on the response to p7.OVA is at least partially dependent on their expression by the same cell

As previously mentioned, a major benefit of gene-gun immunisation is the increased ability to deliver vectors to individual cells. An important question is whether an immunomodulatory gene must be coexpressed by the antigen-expressing cell in order to affect the immune response. The answer to this question may differ depending on the nature of the gene product. Cell surface molecules, intracellular signalling molecules and secreted molecules that have autocrine effects might require coexpression. However, such dependence is less likely for those with paracrine or endocrine effects.

In order to examine the importance of coexpression in this model, cartridge production was modified: Normal cartridge production involves first mixing plasmid vectors at their correct ratio and then coating this mixture onto gold particles. The result is that all gold particles are coated with the same ratio of vectors. In coadministration immunisations of p7.OVA and p7.IL10, both vectors would be found in the same ratio on all particles and would therefore be transfected into the same target cell. This immunisation will be referred to as ‘linked’. In contrast an ‘unlinked’ immunisation involves preparing p7.OVA- and p7.IL10-coated particles separately and mixing them only prior to cartridge preparation. A third treatment will be referred to as ‘adjacent’. In this case, entirely separate cartridges of p7.OVA and p7.IL10 are used. The total amount of DNA and the number of gold particles was constant between all groups. A detailed description of how this was achieved can be found in the Materials and Methods section (2.2.3).

$1x10^6$ DO11.10 cells were adoptively transferred into naïve Balb/c recipients. 24 h later mice were immunised with p7.OVA alone (“Primed”) or with p7.OVA + p7.IL10 in linked, unlinked or adjacent formats. 5 days post-immunisation draining lymph nodes were harvested. DO11.10 proliferation and cytokine expression were examined, as previously.

In agreement with earlier experiments, flow cytometry showed that in mice that received p7.OVA alone, around 3000 per million lymph node cells were $CD4^+KJ1^+$ (Figure 5-8) and that linked p7.IL10 could reduce this to 1000. Interestingly, when p7.IL10 was unlinked, this inhibitory effect was partially lost. A similar loss of effect was seen in mice which received the plasmids on separate cartridges.

ELISpot analysis of the lymph node cells showed a similar pattern in cytokine expression (Figure 5-9). A significant 70% reduction of IL-2- and IL-4-producing cells was caused by coexpressed IL-10. A smaller, 30% reduction was observed when unlinked and adjacent formats were used. The number of IL-10- and IFN-γ-producing cells followed the same pattern across groups.

ELISpot allows the enumeration of cytokine-producing cells at much lower percentages than is normally achieved by flow cytometry. However the exact identity of the cytokine-producers is not provided by this technique. In order to confirm that the $OVA_{323-339}$-dependent ELISpots seen so far were, indeed, produced by adoptively transferred DO11.10 cells, in two experiments an aliquot of lymph node cells was reserved. These cells were stimulated with $OVA_{323-339}$ in the presence of Brefeldin A, fixed, permeablised and intracellularly stained for IL-2. $CD4^+KJ1^+IL\text{-}2^+$ cells were quantified by flow cytometry.

Comparison of ELISpot and intracellular staining (ICS) techniques for the quantification of IL-2 expression shows that they provide very similar information, at least qualitatively (Figure 5-10a). ICS also showed that coexpressed IL-10 reduces the number of IL-2-producing cells and that this effect was partially lost when unlinked and adjacent formats were used. However, the absolute number of IL-2-producers identified by ICS was always roughly one-third of that identified by ELISpot.

Gating on the $CD4^+KJ1^+$ population allowed the examination of IL-2 expression within the DO11.10 population (Figure 5-10b). A very similar pattern was observed: OVA-dependent IL-2 expression by DO11.10 cells was inhibited more greatly by linked than by unlinked or adjacent p7.IL10.

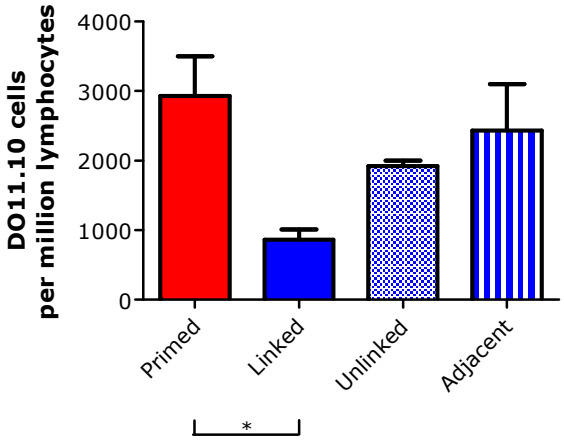



**Figure 5-8. The inhibitory effect of p7.IL10 on the proliferative response to p7.OVA is at least partially dependent on their coexpression by the same cell.**
Following adoptive transfer, mice (3 per group) were immunised with p7.OVA alone ("Primed") or together with p7.IL10 on the same beads ("Linked"), on separate beads ("Unlinked") or in a separate cartridge ("Adjacent"). See Materials and Methods for details. 5 days post-immunisation inguinal lymph nodes were harvested and DO11.10 cells counted by flow cytometry. Data are the mean of four independent experiments.
* $p<0.05$ by one-way ANOVA as compared to "Primed" group

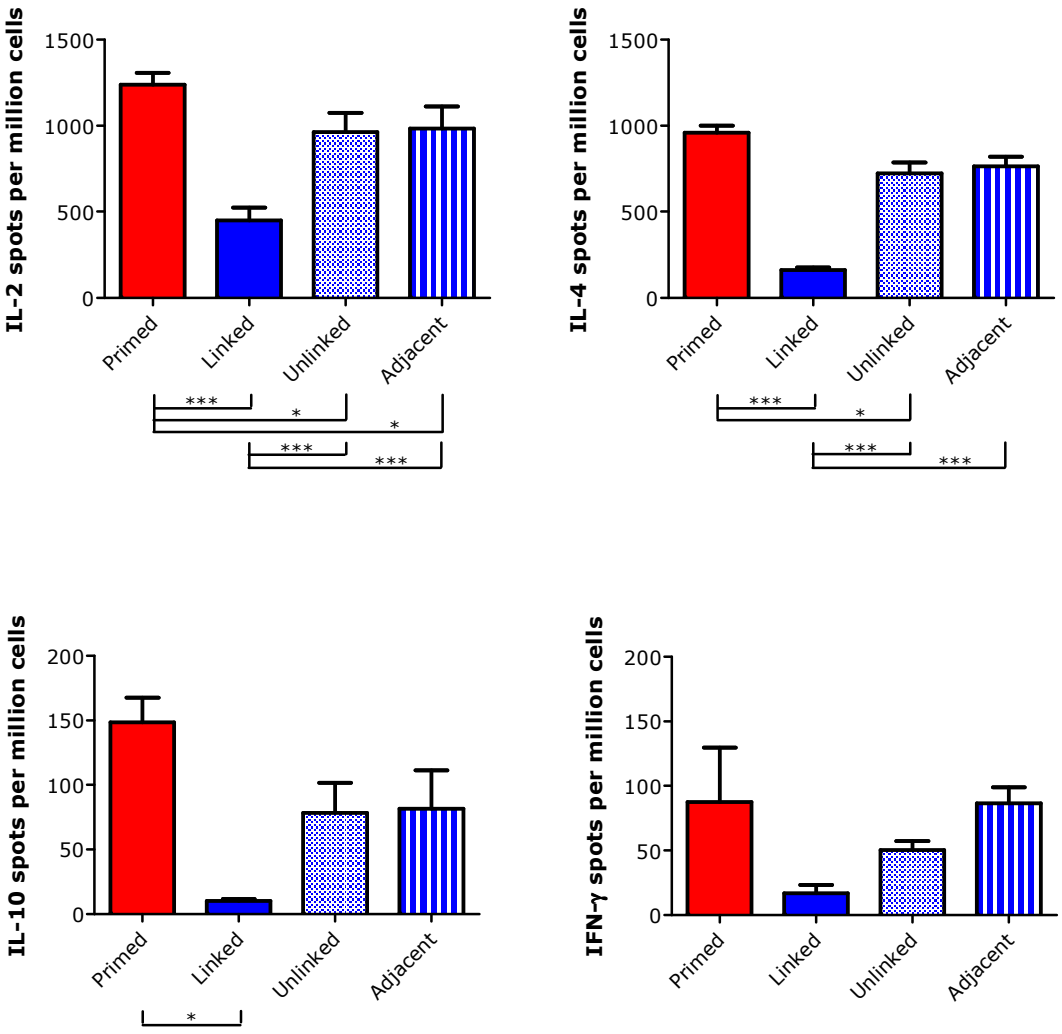



**Figure 5-9. The inhibitory effect of p7.IL10 on the cytokine response to p7.OVA is partially dependent on their coexpression by the same cell.**

Following adoptive transfer, mice (3 per group) were immunised with p7.OVA alone ("Primed") or together with p7.IL10 on the same beads ("Linked"), on separate beads ("Unlinked") or in a separate cartridge ("Adjacent"). See Materials and Methods for details. 5 days post-immunisation inguinal lymph nodes were harvested and the cytokine response analysed by ELISpot. Data are the mean of four independent experiments.

*** $p<0.001$; * $p<0.05$ by one-way ANOVA

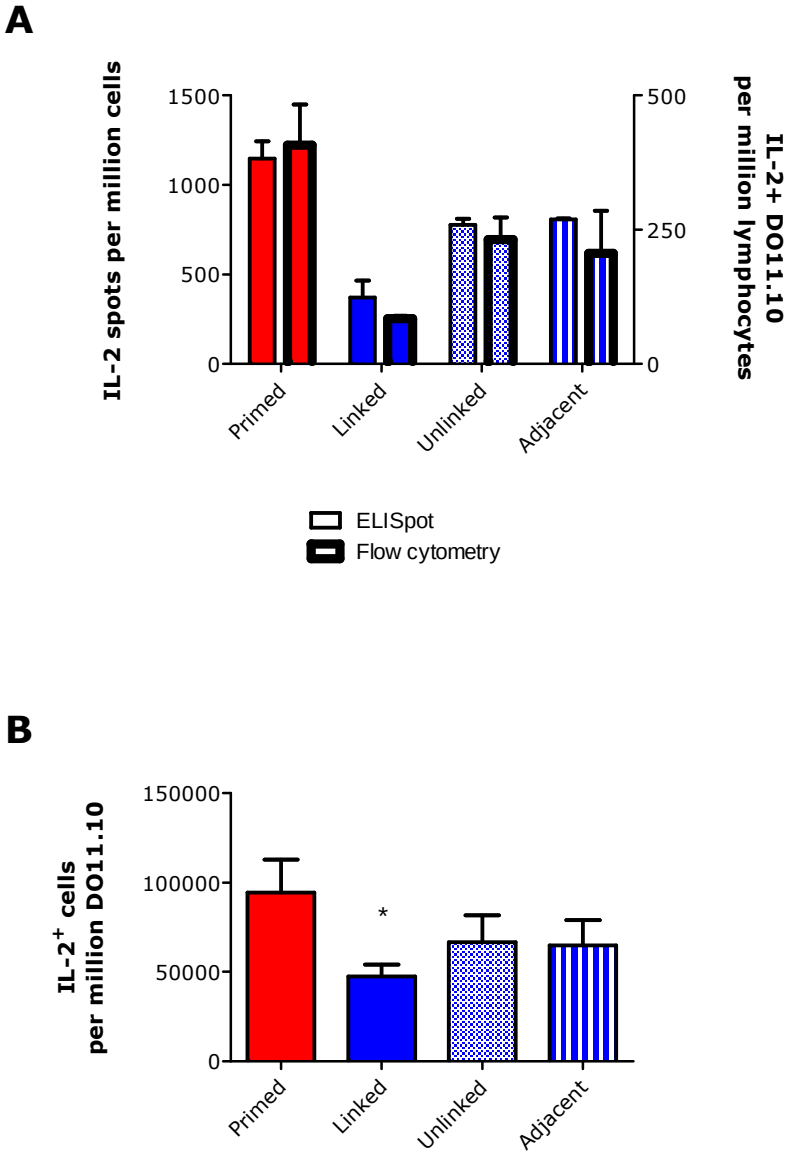



**Figure 5-10. Comparison of IL-2 expression analysis techniques.**
Following adoptive transfer, mice (3 per group) were immunised with p7.OVA alone ("Primed") or together with p7.IL10 on the same beads ("Linked"), on separate beads ("Unlinked") or in a separate cartridge ("Adjacent"). See Materials and Methods for details. 5 days post-immunisation inguinal lymph nodes were harvested. An aliquot of lymph node cells were stained for flow cytometric analysis, as previously, but also fixed, permeablised and stained with anti-IL-2. A) Comparison of ELISPot and intracellular staining for determining overall IL-2 expression. B) Examination of IL-2 expression within the DO11.10 cell subset. Data are the mean of two independent experiments.
* $p<0.05$ by one-way ANOVA compared to 'Primed' group

## 5.3. Discussion

### 5.3.1. p7.IL10 inhibits the response to antigen

The inclusion of p7.IL10 in the immunisation of mice with p7.OVA clearly and consistently inhibited the response of DO11.10 cells to antigen. This effect was dependent on the quantity of p7.IL10 administered, or at least relative to the quantity of antigen-encoding plasmid. This is fully in agreement with the many studies defining IL-10 as an immunosuppressive cytokine.

The number of DO11.10 cells present in the draining lymph nodes was lower in groups that received coadministered p7.IL10 than in those that received p7.OVA alone. The difference between the two groups was exaggerated following a second immunisation. Further analysis, utilising CFSE-staining, showed this effect to correlate with fewer divisions of DO11.10 cells. This observation suggests that reduced proliferation, rather than migration, was the cause of the smaller DO11.10 population.

The cytokine response was also inhibited by p7.IL10 in a dose-dependent manner. When restimulated with $OVA_{323-339}$, fewer lymph node cells from mice that received coadministered p7.IL10 produced IL-2, IL-4, IFN-γ and IL-10 than those from mice that received p7.OVA alone. Again, this inhibition was exaggerated following a second immunisation.

Such a decrease in cytokine-producing cells might be caused purely by the reduced number of total DO11.10 cells found in the lymph node following coadministration of IL-10. However, intracellular staining clearly demonstrated that the ability of DO11.10 cells to produce IL-2 was also inhibited. It would be interesting to examine and compare IL-4 and IFN-γ expression in these cells using the same technique.

Finally, in support of the notion that coadministration of p7.IL10 induces a change in DO11.10 cells, rather than only reducing their number, OVA-induced CD62L downregulation by these cells was inhibited in a p7.IL10-dependent manner. CD62L allows the migration of $CD4^+$ T cells to the secondary lymph node tissues and its downregulation is associated with T cell activation and subsequent migration to the peripheral tissues. That coadministration of p7.IL10 resulted in both fewer DO11.10 cells and less CD62L downregulation suggests that decreased activation, rather than increased emigration, is the cause of the lower number of DO11.10 cells and ELISpots seen in p7.IL10-treated mice.

### 5.3.2. p7.IL10 delays the response to antigen

Examination of the temporal effect on cytokine production by p7.IL10 showed that, although there was significant inhibition at 72 h post-immunisation, after 120 h this difference was much reduced. A similar pattern was observed in the proliferative response to antigen. By staining donor cells with CFSE prior to adoptive transfer it was possible to study in more detail the division of DO11.10 cells. Again, the difference between mice that received p7.OVA alone and those that received coadministered p7.IL10 was greatest 72 h post-immunisation. By 96 h the number of cell divisions in the latter group had begun to approach the number in the former.

Effectively, the response by DO11.10 cells to immunisation with p7.OVA was delayed by the coadministration of p7.IL10. One feasible cause of this effect is the relative persistence of the two gene products. Whereas ovalbumin is a structural and/or storage protein, IL-10, as a cytokine, is typically short-lived. In this system both genes are expressed via the same promoter but, after expression has ceased, it is possible that ovalbumin persists for longer than IL-10. Additionally, immunogenic OVA peptides may continue to be presented on MHC molecules after intact ovalbumin has been cleared. Continued antigen presentation in the absence of IL-10 might overcome any previous inhibition. Whilst taking into account this caveat, other evidence agrees that IL-10 may, in fact, delay the response to antigen[(Bai *et al.*, 1997; Zhao *et al.*, 2005)].

As in 5.2.3, in order to examine less transient effects caused by coadministration of p7.OVA most future experiments examined lymph node cells 5, as opposed to 3, days post-immunisation.

### 5.3.3. Dependence on linkage

Examination of linkage in the above manner assumes that most, if not all, transfected cells receive only one bead. This is a complex issue involving both the initial coating of the beads and the distribution of beads in the transfected tissue. However, the evidence is certainly in favour of this occurrence:

Firstly, previous work has shown that the procedure for combining separately coated beads in the same cartridge is, indeed, effective[(Creusot, 2002)]. In addition, because only a small proportion of DC are transfected by PMDD it is likely that only one bead will enter an individual cell[(Porgador *et al.*, 1998)]. Secondly, on a macro scale, unlinked and adjacent immunisations result in a similar response. The latter is certainly 'unlinked' in that the individual p7.OVA and p7.IL10 shots are spacially separated. The literature and data presented here both, therefore, suggest that the unlinked immunisation does, indeed, transfer the two plasmids to separate cells.

The response of T cells to antigen is known to be dependent on the microenvironment at the time of presentation by APC. This allows T cells of the same or different antigen specificity to enhance (cooperation[(Kalams and Walker, 1998; Gerloni *et al.*, 2000; Creusot *et al.*, 2003b)] or synergy[(Wang *et al.*, 2001; Mintern *et al.*, 2002)]), modify[(Schuhbauer *et al.*, 2000; Creusot *et al.*, 2003a)] or diminish (inhibition[(Jonuleit and Schmitt, 2003)] or competition[(Laouar and Crispe, 2000; Smith *et al.*, 2000)]) the response of other T cells in the same DC cluster.

The use of the adoptive transfer / gene gun model to dissect this mechanism has previously shown that *in vitro*-polarised T cells can influence naïve T cells of different antigen specificity if antigens are linked i.e. their genes are located on the same microprojectile during immunisation[(Creusot *et al.*, 2003a)]. Using this same model, this study has shown that the requirement for linkage is also true for the inhibitory effect of IL-10 on the response: The separation of p7.OVA and p7.IL10 onto different microprojectiles reduced the effect of the latter on the response to the former by half.

Professional APC such as DC are able to uptake and present antigen from their surroundings (referred to here as antigen transfer). It is generally understood that antigen processed in this manner is presented on MHC class II molecules, whilst endogenously synthesised antigen is presented by most cells, including professional and non-professional APC, on MHC class I molecules. However, DC are

also able to pass exogenous antigen into the MHC class I pathway for presentation to $CD8^+$ T cells; a mechanism known as cross-priming[(Bevan, 2006; Shen and Rock, 2006; Basta and Alatery, 2007)]. Cross-priming has been demonstrated to be vital in the generation of not only the $CD8^+$ but also the $CD4^+$ T cell response in a model similar to ours[(Lauterbach *et al.*, 2006)]. In addition, peptides from endogenous, often membrane-bound, proteins have been isolated from MHC class II complexes[(Rudensky *et al.*, 1991; Chicz *et al.*, 1993)]. The question of which antigens are processed by which MHC pathway is clearly complex and is the subject of extensive studies[(Juncker *et al.*, 2009)].

The markedly decreased effect of p7.IL10 in an unlinked format suggests that the separation of plasmids and/or gene products is maintained, at least to some degree, following immunisation. Directly transfected APC as the main source of presented antigen would cause presentation to occur away from unlinked p7.IL10. Conversely, if antigen transfer was central to the response, this would imply secretion and uptake of antigen; spatial separation of OVA and IL-10 would be greatly reduced and thus linkage would be less important. This agrees with the DC cluster model of Creusot *et al*. whereby DC present antigen in a distinct microenvironment created by both the DC and T cells under minimal influence of other clusters[(Creusot *et al.*, 2002)]. It would be reasonable to suggest, therefore, that at least a significant proportion of antigen presentation in this model is performed by directly transfected DC.

A caveat that will not be fully addressed here is the skewed view of the immune response given by the use of the MHC class II-specific DO11.10 cell. Because the TCR of this $CD4^+$ T cell only recognises the $OVA_{323-339}$ epitope in the context of the MHC class II molecule, data is confined to this part of the response only. This is particularly important to note when studying an antigen that is assumed to be, to some degree at least, synthesised and presented by the same cell: Despite the complexity of the antigen processing pathways (described above) it might be expected that the primary presentation would still occur via the MHC class I pathway. Therefore results should be interpreted as being viewed through a particular window and not as a description of the response as a whole. Nevertheless, the dependence of IL-10 on linkage is clear.

In conclusion, this chapter validates that IL-10 can be delivered via PMDD and that it will exert an immunosuppressive effect in a very localised manner. It seems reasonable to hypothesise that the microenvironment of this IL-10 effect is, in fact, the DC-T cell cluster.

# 6. Induction of Tolerance

## 6.1. Introduction

Although inclusion of the IL-10-encoding plasmid p7.IL10 has been shown to reduce the response to immunistion with p7.OVA it is important to understand whether the DO11.10 cells involved retain any memory of this treatment. A reduced response may be due to one or more of three possible reasons: 1) The presence of IL-10 inhibits antigen presentation and nonresponsive cells remain naïve[(de Waal Malefyt *et al.*, 1991b; Koppelman *et al.*, 1997; Redpath *et al.*, 1999; Faulkner *et al.*, 2000)]. 2) The T cell is anergised during treatment and its response to subsequent antigen challenge is less vigorous than it otherwise would have been[(Groux *et al.*, 1996; Steinbrink *et al.*, 1997; Kubsch *et al.*, 2003; Beinhauer *et al.*, 2004)]. 3) The cell becomes a regulatory T ($T_R1$) cell, modulating the response of neighbouring cells to subsequent antigen challenge[(Takayama *et al.*, 1999; Akbari *et al.*, 2001; Henry *et al.*, 2008; Zhu *et al.*, 2008a)]. There is evidence, however, that anergised T cells are, in fact, regulatory[(Buer *et al.*, 1998; Steinbrink *et al.*, 2002)].

This chapter concentrates on elucidating which of these mechanisms is/are responsible for the previously observed inhibition by p7.IL10 and determining if the effect is long-lasting i.e. if tolerance is induced.

## 6.2. Results

The basic model used in both this and the next chapter is that of prime-boost-challenge. In this chapter the prime and boost immunisations were effectively the same as those of the double-dose studies in the last chapter. Two weeks after the latter of these immunisations mice received a third immunisation (the 'challenge') with either p7.OVA alone or with the control p7 vector.

### 6.2.1. Coadministration of p7.IL10 during two rounds of immunisation reduces the response to subsequent challenge with antigen

Figure 6-1 shows that a prime-boost treatment followed by further challenge with p7.OVA significantly enhances the number of OVA-specific IL-2- and IL-4-producing cells in the lymph nodes compared to the control groups. Coadministration of p7.IL10 in the prime and boost immunisations reduced the number of cytokine-secreting cells in response to the challenge with p7.OVA. A similar pattern of stimulation and inhibition was seen at both 72 h and 120 h post-challenge but with roughly twice as many cytokine ELISpots in every group at the later timepoint.

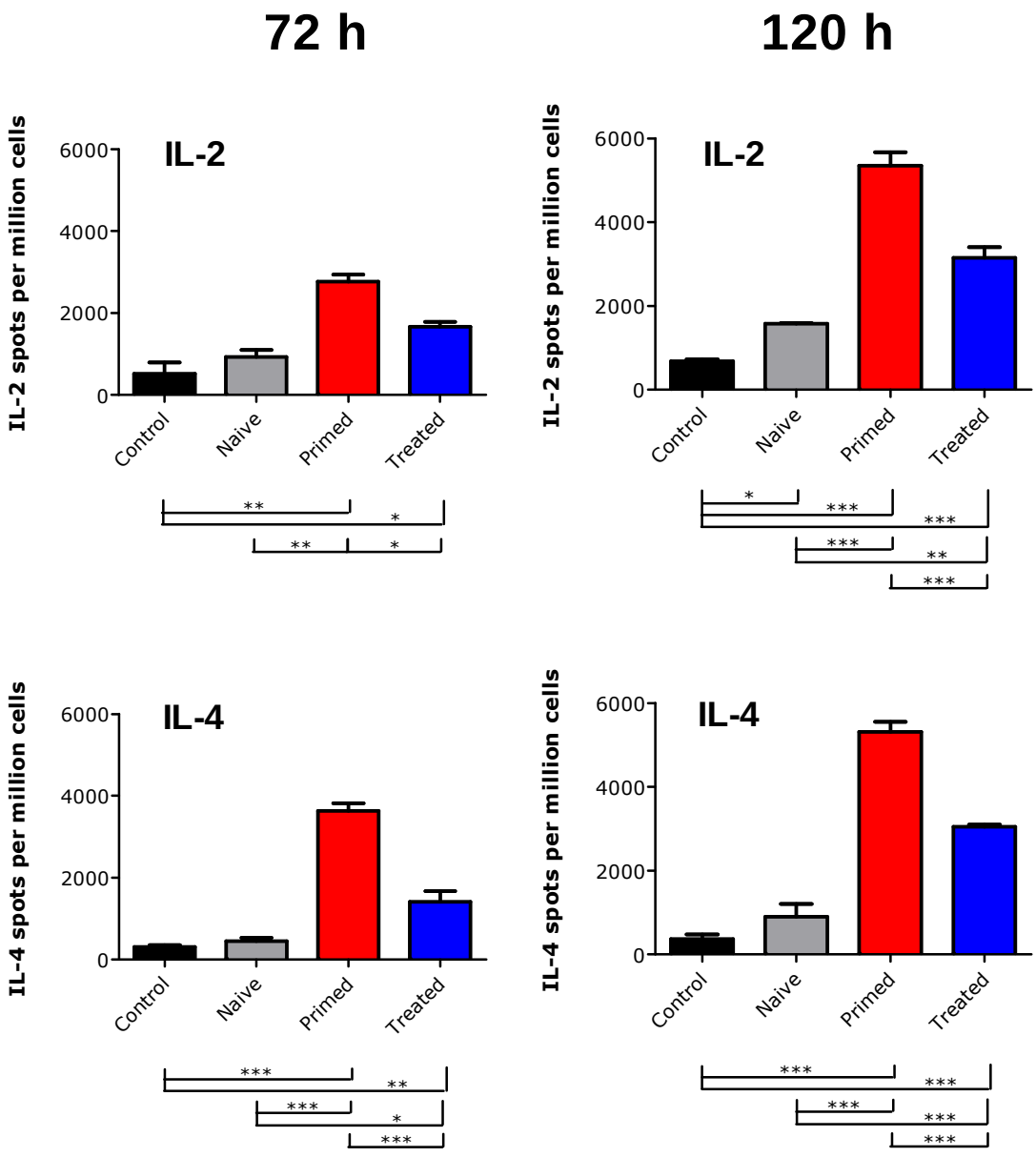



**Fig 6-1 Coadministration with p7.IL10 during prime-boost reduces the number of cytokine-producing cells in the lymph node in response to later challenge with p7.OVA.**

Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised ('prime') with either empty p7 ("control" and "naïve"), p7.OVA ("primed") or p7.OVA + p7.IL10 ("treated"). Two weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were immunised with p7.OVA ('challenge'); the control group received empty p7 vector instead. 3 or 5 days post-challenge inguinal lymph nodes were harvested. IL-2 and IL-4 ELISpots were performed as previously described, using 1 µM $OVA_{323-339}$ peptide. Data are the mean of three independent experiments.

*** p<0.001; ** p<0.01; * p<0.05

### 6.2.2. Inhibition by p7.IL10 of the cytokine response to p7.OVA is dependent on adoptive transfer of DO11.10 cells

Data in earlier chapters, that employed shorter immunisation protocols, was not replicable in Balb/c mice that had not received adoptively transferred DO11.10 cells because the small number of antigen-specific T cells did not allow ELISpot or flow cytometric analysis; indeed, this is a key reason for the use of the adoptive transfer model in the first place. The three immunisations in the current protocol increase the number of endogenous antigen-specific T cells to a number detectable by ELISpot.

Prime-boost with p7.OVA produced a detectable, and statistically significant, number of antigen-specific, IL-2- and IL-4-producing T cells (Figure 6-2). In contrast to Figure 6-1, in the absence of DO11.10 cells the coadministration of p7.IL10 did not reduce the number of cytokine ELISpots produced in response to p7.OVA challenge.

The endogenous response to OVA will likely involve epitopes other than 323-339. In order to understand the importance of the other epitopes in this system, cytokine ELISpots were performed using whole ovalbumin protein at the same molar concentration (Figure 6-3). The overall pattern was the same as that for peptide ELISpots: Prime-boost with p7.OVA increased the number of cytokine ELISpots and coadministration of p7.IL10 failed to significantly inhibit this stimulation (Figure 6-3a&b). As might be expected, the absolute number of ELISpots was higher in every case when whole protein was used.

Interestingly, the ratio of 323-339:other epitope ELISpots was different for each cytokine. Only half of the IL-2 ELISpots were attributable to the 323-339 epitope whilst roughly 70% of IL-4 spots were (Figure 6-3 c&d).

### 6.2.3. The pool of primed antigen-specific T cells is lower in mice that are primed with p7.OVA+p7.IL10 than in mice that receive p7.OVA alone

One of the reasons for the experiments in section 6.2.1 was to dissect the direct effect of the immunisation on naïve T cells from any indirect downstream effects that might be caused by these changes. To understand the immune situation at the time of the final antigenic challenge, rather than administer the challenge immunisation, spleens were examined two weeks post-boost.

The number of DO11.10 cells in the spleen was roughly doubled in mice that received a prime-boost of p7.OVA alone compared to controls (Figure 6-4). This increase was completely abrogated by the inclusion of p7.IL10 in the immunisation. Interestingly, the large increase in DO11.10 number corresponded to only a small increase in the number of IL-2-producing cells (Figure 6-5). In contrast, the inhibition caused by coadministration of p7.IL10 was, indeed, reflected by a highly significant reduction in the number of IL-2-producers. No significant effect was seen on the number of IL-4-producers, either by p7.OVA or by p7.OVA+p7.IL10.

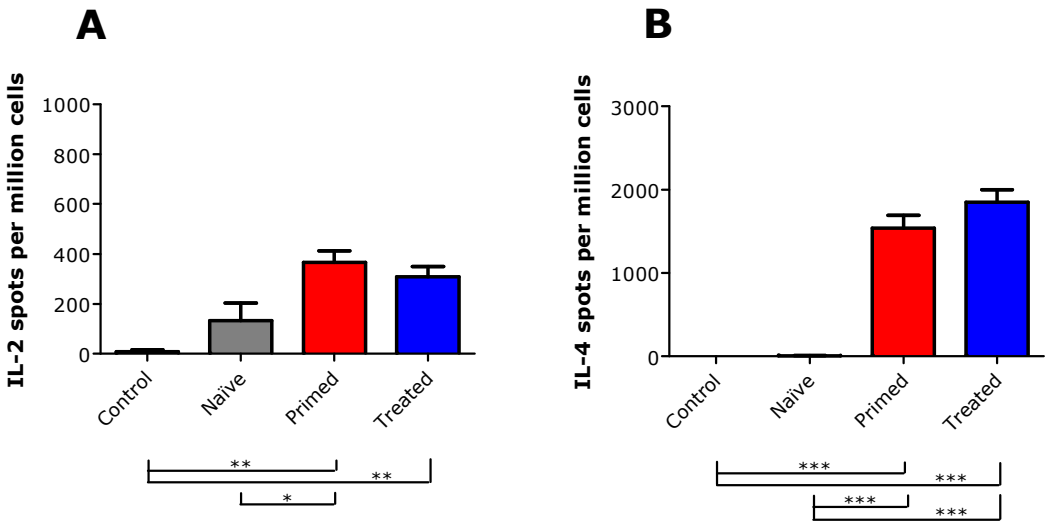



**Figure 6-2. In the absence of adoptively transferred DO11.10 cells coadministration with p7.IL10 during prime-boost has no effect on the number of $OVA_{323-339}$-specific cytokine-producing cells in the lymph node in response to later challenge with p7.OVA.**

Balb/c mice (3 per group) were immunised ('prime') with either empty p7 ("control" and "naïve"), p7.OVA ("primed") or p7.OVA + p7.IL10 ("treated"). Two weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were immunised with p7.OVA ('challenge'); the control group received empty p7 vector instead. 3 days post-challenge inguinal lymph nodes were harvested. IL-2 and IL-4 ELISpots (A and B, respectively) were performed as previously described, using 1μM $OVA_{323-339}$ peptide.

*** $p<0.001$; ** $p<0.01$; * $p<0.05$ by one-way ANOVA

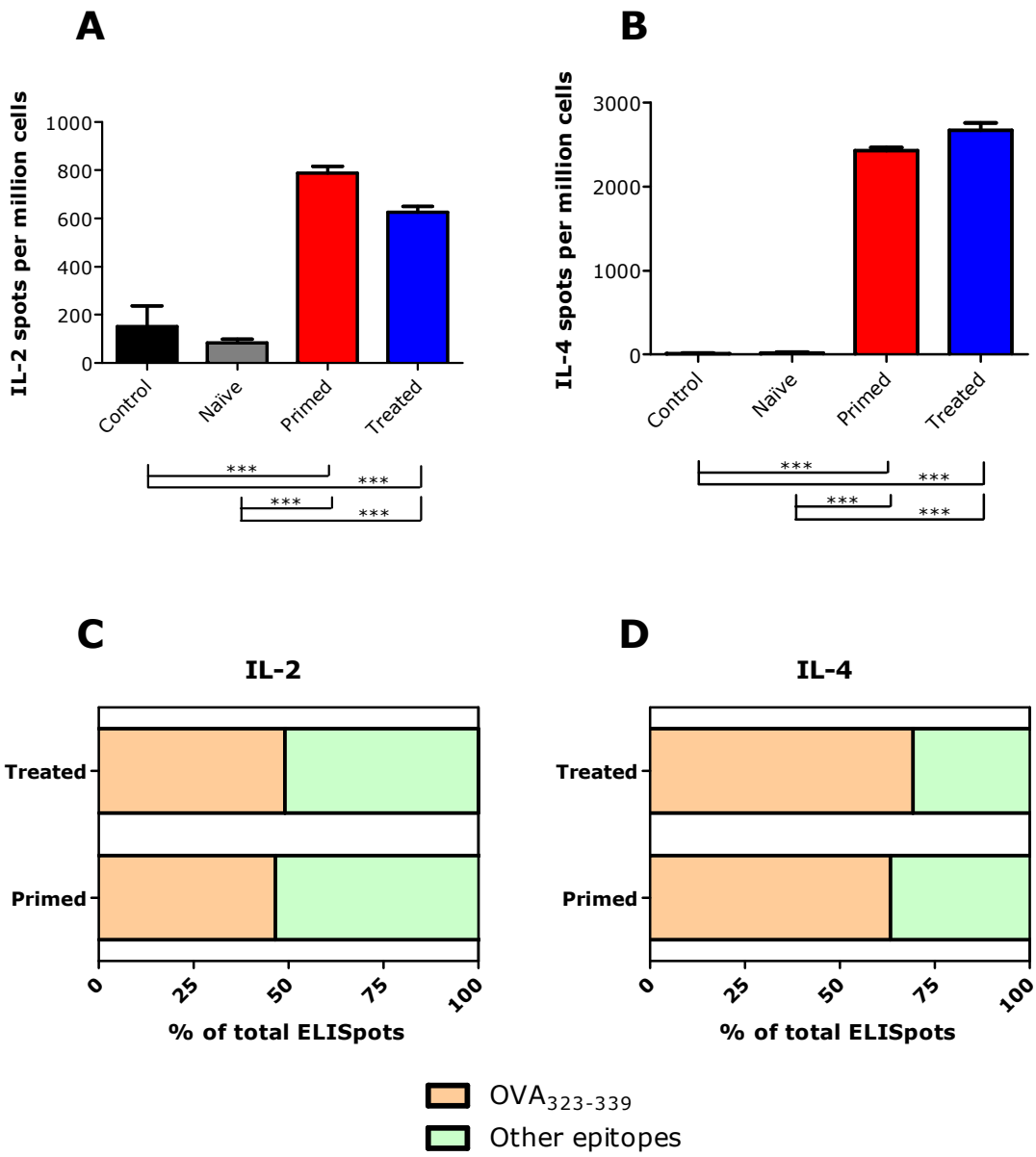



**Figure 6-3. In the absence of adoptively transferred DO11.10 cells coadministration with p7.IL10 during prime-boost has no effect on the number of ovalbumin-specific cytokine-producing cells in the lymph node in response to later challenge with p7.OVA. Other epitopes are more $T_H2$-biased than is the 323-339 epitope.**

Balb/c mice (3 per group) were immunised ('prime') with either empty p7 ("control" and "naïve"), p7.OVA ("primed") or p7.OVA + p7.IL10 ("treated"). Two weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were immunised with p7.OVA ('challenge'); control groups received empty p7 vector instead. 3 days post-challenge inguinal lymph nodes were harvested. IL-2 and IL-4 ELISpots (A and B, respectively) were performed as previously described, but using 1μM ovalbumin protein as the stimulating antigen.

Data were compared to the corresponding ELISpots that utilised $OVA_{323-339}$ peptide and are displayed as the percentage of cytokine ELISpots produced in response to the 323-339 epitope vs those produced in response to other epitopes (C and D).

*** $p<0.001$ by one-way ANOVA

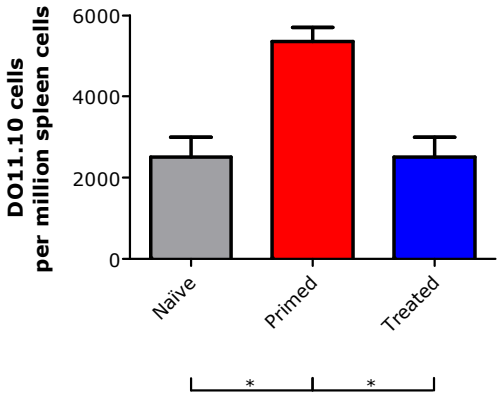



**Figure 6-4. Coadministration with p7.IL10 during prime-boost reduces the size of the pool of DO11.10 cells in the spleen.**

Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised (=prime) with either empty p7 ("naïve"), p7.OVA ("primed") or p7.OVA + p7.IL10 ("treated"). Two weeks later mice were re-immunised with the same vector (=boost). Two weeks post-boost spleens were harvested. Spleen cells were examined by flow cytometry as previously described to quantify the number of DO11.10 cells present. Data are the mean of two independent experiments.

* $p<0.01$ by one-way ANOVA

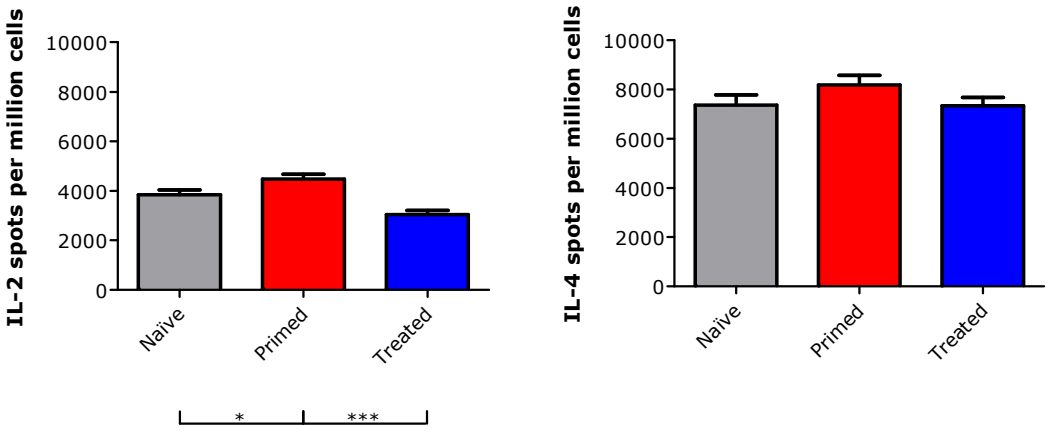



**Figure 6-5. Coadministration with p7.IL10 during prime-boost reduces the size of the pool of cytokine-producing cells in the spleen.**
Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised (=prime) with either empty p7 ("naïve"), p7.OVA ("primed") or p7.OVA + p7.IL10 ("treated"). Two weeks later mice were re-immunised with the same vector (=boost). Two weeks post-boost spleens were harvested. Cytokine production by spleen cells were examined by IL-2 and IL-4 ELISpot (A and B, respectively). Data are the mean of two independent experiments.
*** $p<0.001$; * $p<0.01$ by one-way ANOVA

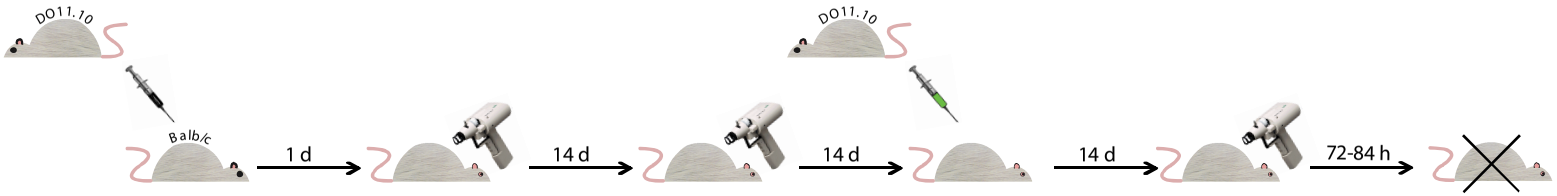
### 6.2.4. Host antigen experience, and not the coadministration of p7.IL10, determines the proliferative capacity of freshly-transferred DO11.10 cells

To further examine the possibility that DO11.10 cells were tolerised their ability to affect the activation of naïve T cells was examined. An *in vivo* approach was devised whereby a second adoptive transfer of DO11.10 cells was administered following prime-boost treatment. These fresh DO11.10 cells were labeled with CFSE prior to transfer, firstly to distinguish them from cells from the initial transfer and secondly to examine their proliferative response to the final challenge with p7.OVA (Figure 6-6).

Two control groups received a final challenge with empty p7 vector. One of these had previously received prime-boost immunisations of p7 and the other had received p7.OVA. Importantly, irrespective of earlier immunisations, CFSE-stained T cells in both groups exhibited no proliferation (Figure 6-7). This shows that previously activated T cells cannot induce proliferation in naïve T cells in the absence of antigen and that there is no residual antigen presentation two weeks after gene gun immunisation.

72 h post-challenge proliferation was seen in all groups whose challenge immunisation contained p7.OVA. Interestingly, the degree of proliferation was the same in all groups. Over the next 12 hours, however, the total number of divisions in those groups that had previously experienced antigen increased by 150-200% whilst the number in previously naïve mice increased by 300-450%. A key observation is that the presence of p7.IL10 in prime and boost immunisations had no effect on the proliferation of CFSE-stained DO11.10 cells.

The number of $CFSE^{-}$ DO11.10 cells was also counted at 72 h and 84 h post-challenge (Figure 6-7). In this case control groups which received no second adoptive transfer, and therefore could not be examined as above, were included. In most groups, $CFSE^{-}$ DO11.10 comprised roughly 5000 per million lymphocytes at 72 h post-challenge and 10,000 per million after 84 h. The largest anomaly from this pattern was observed in mice that received prime-boost immunisations of empty p7 vector: Elimination of the second adoptive transfer resulted in double the number of $CFSE^{-}$ DO11.10 compared to mice that did receive the CFSE-stained DO11.10 cells.




| **"Adoptive transfer"** 1x10$^6$ DO11.10 cells into Balb/c recipients | **"Prime"** PMDD immunisation | **"Boost"** PMDD immunisation | **"CFSE adoptive transfer"** 1x10$^6$ DO11.10 cells into Balb/c recipients | **"Challange"** PMDD immunisation | **"Analysis"** Lymph nodes harvested for flow cytometry |
|---|---|---|---|---|---|
| ✓ | p7 | p7 | ✓ | p7.OVA | ✓ |
| ✓ | p7 | p7 | | p7.OVA | ✓ |
| ✓ | p7.OVA | p7.OVA | ✓ | p7.OVA | ✓ |
| ✓ | p7.OVA | p7.OVA | | p7.OVA | ✓ |
| ✓ | p7.OVA + p7.IL10 | p7.OVA + p7.IL10 | ✓ | p7.OVA | ✓ |
| ✓ | p7.OVA + p7.IL10 | p7.OVA + p7.IL10 | | p7.OVA | ✓ |
| ✓ | p7 | p7 | ✓ | p7 | ✓ |
| ✓ | p7.OVA | p7.OVA | ✓ | p7 | ✓ |

**Figure 6-6. The CFSE challenge model**
In order to identify any inhibitory effect on naive T cells fresh, CFSE-stained DO11.10 were adoptively transferred following prime and boost immunisations. 1x10$^6$ DO11.10 cells were transferred into Balb/c recipients. 1 day later mice were immunised ("prime") with empty p7, p7.OVA or p7.OVA+p7.IL10. Two weeks later mice received a second immunisation ("boost") with the same vector(s). Two weeks post-boost mice received a second adoptive transfer of 1x10$^6$ naive DO11.10 cells which, this time, had been stained with 5µm CFSE. Control mice received no adoptive transfer. 24 hours later mice received a challenge immunisation of p7.OVA. Control mice received empty p7 vector instead. 72-84 h later, mice were sacrificed, lymph nodes harvested and lymph node cells examined by flow cytometry.

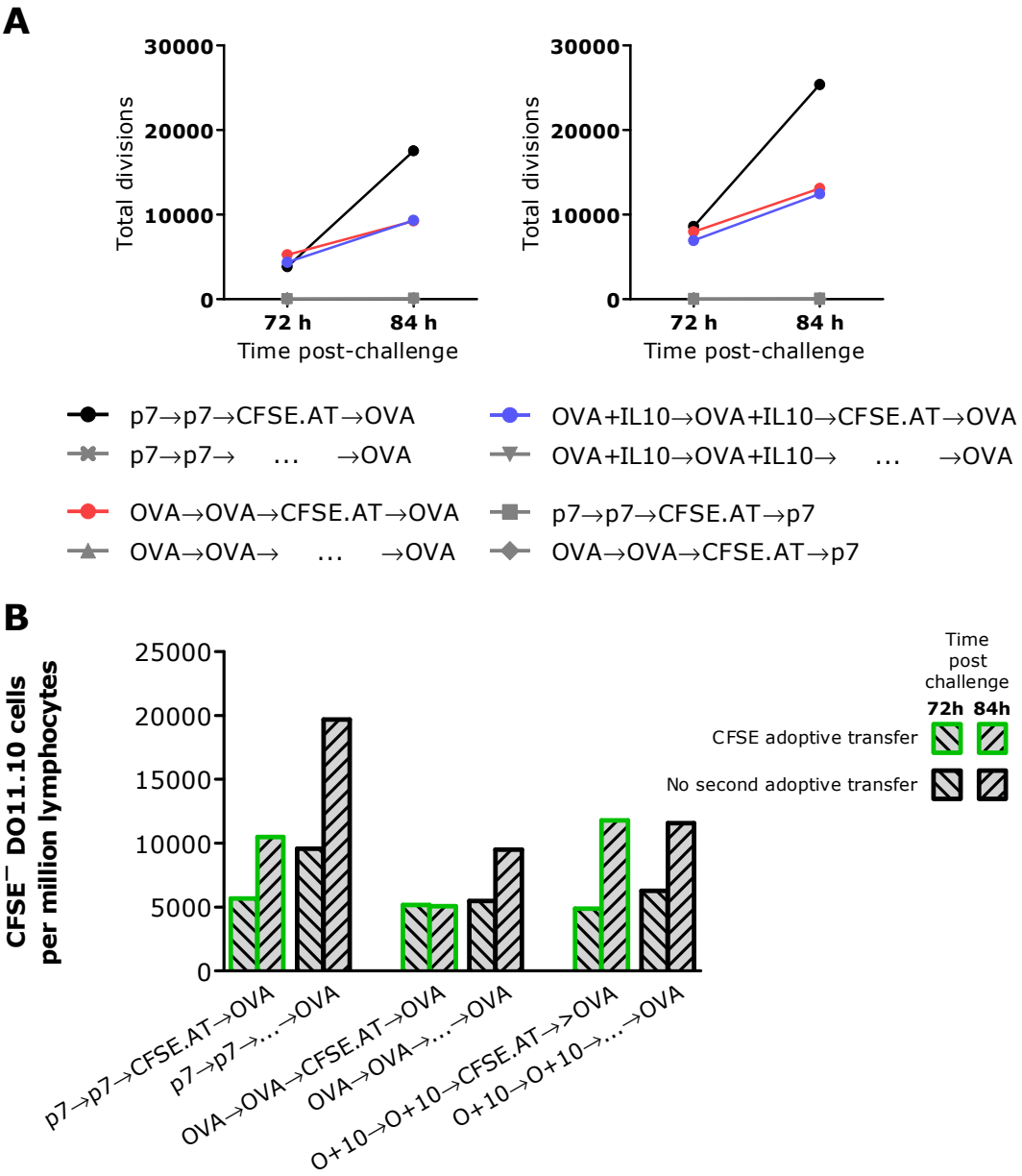



**Figure 6-7. Host antigen experience, and not the coadministration of p7.IL10, determines the proliferative capacity of freshly-transferred DO11.10 cells.**

Balb/c mice (3 per group) were adoptively transferred with 1x10$^6$ DO11.10 T cells. 1 day later mice were immunised ("prime") with empty p7, p7.OVA or p7.OVA+p7.IL10. Two weeks later mice received a second immunisation ("boost") with the same vector(s). Two weeks post-boost mice received a second adoptive transfer of 1x106 naive DO11.10 cells which, this time, had been stained with 5μm CFSE. Control mice received no adoptive transfer. 24 hours later mice received a challenge immunisation of p7.OVA. Control mice received empty p7 vector instead. See Figure 6.6 for full details. 72 h and 84 h post-challenge mice were sacrificed and inguinal lymph nodes harvested. Lymph node cells were stained and examined by flow cytometry: CD4$^+$KJ1.26$^+$ (DO11.10) cells were gated and CFSE fluorescence was examined.

(A) Calculated total cell divisions based on CFSE fluorescence peaks (see Materials & Methods for details); graphs show data from two independent experiments. (B) Frequency of CFSE$^-$ cells in the same draining lymph nodes; data are representative of two independent experiments.

## 6.3. Discussion

### 6.3.1. The inhibitory effect of p7.IL10 has long-term ramifications for later antigen challenge

The inclusion of p7.IL10 in prime and boost immunisations clearly resulted in fewer cytokine-producing cells in the draining lymph nodes (Figure 6-1). This could be interpreted as inhibition of DO11.10 cells to future stimulation; either anergy or tolerance. However, earlier experiments showed that a double immunisation with p7.OVA+p7.IL10 results in a smaller number of cytokine-producing DO11.10 cells in the draining lymph nodes (Figures Figure 5-6 and Figure 5-7) and examination of the spleen at the time of the challenge immunisation confirms that this reduction is mirrored in the general T cell pool (Figure 6-4 and Figure 6-5). Therefore the reduced number of cytokine-producing cells in the "treated" group may reflect the smaller number of DO11.10 at the time of challenge. At no time was a lower number of cytokine-producing cells detected in the IL-10-treated group than in the "naïve" group. Therefore classical tolerance was not observed.

### 6.3.2. The inhibitory effect of p7.IL10 is dependent on adoptively transferred DO11.10 cells.

Interestingly, the coadministration of p7.IL10 in prime and boost immunisation had no significant effect on the response to challenge with p7.OVA if mice did not initially receive adoptively transferred DO11.10 cells (Figure 6-2 and Figure 6-3). Earlier experiments with shorter experimental protocols could not be performed without adoptive transfer because the number of responder cells was too low; indeed, this is a reason for introducing adoptive transfer in the first place. Several possible explanations for this observation exist:

Are epitope 323-339-specific T cells easier than others to inhibit or tolerise? There is no evidence in the literature to suggest that certain epitopes might be more or less inherently tolerogenic than others. In addition, comparison of Figure 6-2 and Figure 6-3 shows that whole ovalbumin-stimulated ELISpot exhibited a similar degree of inhibition as did $OVA_{323-339}$ peptide-stimulated ELISpot. It is also unlikely that the physical transfer of DO11.10 cells makes them more susceptible to inhibition or tolerisation. A more feasible explanation may be that an increased absolute number of naïve antigen-specific T cells allows a sufficient number to be exposed to antigen and IL-10 and therefore tolerised and/or merely allows the effects of IL-10 to be more clearly observed due to the higher number of total ELISpots.

Another explanation might be connected to the importance of 'linkage' of OVA- and IL-10-encoding vectors and, therefore, of directly-transfected DC (see 5.2.4). If the frequency of directly-transfected DC is low compared to the number which present captured OVA rare, endogenous OVA-specific T cells are less likely to encounter these key immunosuppressive cells than are populous, adoptively transferred DO11.10 cells.

### 6.3.3. The $OVA_{323-339}$ epitope may have intrinsic $T_H2$ properties

Information on the relative quantities of epitope-specific T cells could only be supplied by experiments that did not involve adoptive transfer. Specifically, it was possible to compare the number of ELISpots produced in response to restimulation with $OVA_{323-339}$ peptide by those to whole ovalbumin protein. It was only possible to examine data from those groups that exhibited significant numbers of ELISpots i.e. those that received prime and boost with p7.OVA either alone or with p7.IL10 ("Primed" and "Treated", respectively). Interestingly, the data from both groups suggest that the 323-339 epitope itself has a $T_H2$ predisposition: It is responsible for roughly half of IL-2 ELISpots but almost 70% of IL-4 ELISpots. This observation was made late in the project and was not investigated further.

### 6.3.4. p7.IL10 does not induce 'infectious tolerance' such as regulatory T cells

The double-adoptive transfer experiments using CFSE-stained cells were intended to identify any regulatory effect, induced by coadministration of p7.IL10, that might inhibit a response by naïve DO11.10 T cells to a challenge with p7.OVA. That there was no observable difference between mice that received prime-boost of p7.OVA and those that received p7.OVA+p7.IL10 demonstrates that no prolonged inhibitory effect is confered.

In contrast it appears that any prior exposure to antigen, with or without IL-10, reduced the proliferative capacity of freshly transferred DO11.10 cells. Mice that received prime-boost with empty p7 vector exhibited twice as much CFSE-stained DO11.10 cell division than those immunised with p7.OVA or p7.OVA+p7.IL10. This suggests an inhibitory effect by all antigen-experienced T cells. This latter explanation is supported by the data in Figure 6-7b where the inverse effect is seen: The expansion of the initially transferred, unstained, DO11.10 is greater when there is no secondary adoptive transfer, implying that there are, indeed, limited resources, such as antigen or cytokine, for which the two populations compete.

### 6.3.5. Competition and inhibition?

The responses of DO11.10 cells freshly transferred into "treated" (p7.OVA+p7.IL10) mice showed similar inhibition to subsequent antigenic challenge as those transferred into "primed" (p7.OVA) mice. However, it has already been shown that "treated" mice contain far fewer DO11.10 cells than do those in the "primed" group. There is therefore a hint that cells in "treated" mice are more efficient at inhibiting naïve cells than are those in "primed" mice. This might suggest that the "treated" group contains regulatory T cells. However the data as they stand support the notion that, in this model, competition, not active suppression, is the major mechanism whereby antigen experience diminishes activation of naïve T cells.

# 7. The effect of IL-10 on antigen-induced asthma

## 7.1. Introduction

Asthma can be defined as an inflammation of the airways associated with airway hyperresponsiveness and mucous hypersecretion[(Busse and Lemanske, 2001)]. However it is a complex condition and its aetiology and pathology is not always congruous between individuals or between humans and and animal models[(Anderson, 2008)]. It is therefore difficult to extrapolate conclusions from a particular animal model to a prediction of outcome in clinical disease. Nevertheless, in itself, the ovalbumin-dependent asthma model in this chapter can serve as a useful tool in examining the initiation of allergy and allergen-induced asthma.

### 7.1.1. Asthma - a $T_H2$ disorder?

Allergen-induced asthma appears to be associated with, if not mediated by, an increased number of $T_H2$ cells and elevated levels of $T_H2$ cytokines in the lung tissue[(Robinson *et al.*, 1992)]. Indeed, IL-4-deficient mice exhibit a much milder inflammation in terms of both lung histology and eosinophil infiltration[(Brusselle *et al.*, 1994)]. $T_H2$ cytokines play such an important role that merely administering IL-4 or IL-13 is sufficient to cause asthma[(Grunig *et al.*, 1998; Wills-Karp *et al.*, 1998; Kuperman *et al.*, 2002)].

Clinical data in agreement with this include two studies which show in vitro stimulation of cells can distinguish atopic/allergic individuals from healthy; the former producing $T_H2$ cytokines whilst the latter produce a mixture of $T_H1$, $T_H2$ and IL-10[(Akdis *et al.*, 2004; Tiemessen *et al.*, 2004)].

The reciprocal relationship between $T_H1$ and $T_H2$ cells and cytokines has been known for some time[(Mosmann and Coffman, 1989; Street and Mosmann, 1991; Abbas *et al.*, 1996)]. Antigen-specific $CD4^+$ T cells isolated from from atopic individuals have been shown to produce IL-4 in response to Ag whilst those from normal individuals produce IFN-γ[(Wierenga *et al.*, 1990)]. Particularly relevant to this model of atopic asthma, IFN-γ and IL-4 have been shown to inhibit and promote, respectively, production of IgE by plasma cells[(Snapper and Paul, 1987; Pene *et al.*, 1988)].

A series of studies by Cohn *et al*. elegantly demonstrated the relationship between $T_H1$ and $T_H2$ cells in asthma: Adoptive transfer of $T_H2$-primed DO11.10 cells before administration of aerosolised ovalbumin dramatically increased airway inflammation, mucous production and eosinophilia compared to that induced by naïve DO11.10 cells[(Cohn *et al.*, 1997)]. $T_H1$ cells allowed inflammation but did not induce increased mucous production or eosinophilia. Cotransfer of these cells reduced, in an IFN-γ-dependent manner, the level of mucous production and eosinophilia caused by transfer of $T_H2$ cells alone[(Cohn *et al.*, 1999)].

Many studies have attempted to use this relationship to treat asthma. Early work showed that administration of $T_H1$ cytokines, either systemically or directly to the target tissue, could abrogate disease[(Iwamoto *et al.*, 1993; Gavett *et al.*, 1995; Lack *et al.*, 1996)]. Infection with BCG, a known $T_H1$-inducing microorganism, was also able to reduce inflammation and eosinophilia in the lung[(Erb *et al.*, 1998)].

However, this $T_H1/T_H2$ paradigm may be an oversimplification, at least in human disease; many allergic individuals produce a mixture of $T_H1$ and $T_H2$ cytokines to allergens[(Li *et al.*, 1998; Randolph *et al.*, 1999a; Randolph *et al.*, 1999b; Magnan *et al.*, 2000)].

Despite the studies described above, it has become understood that human asthma is far more complex than a symptom of atopy and certainly more complex than the original models of allergic airway inflammation[(Wenzel and Holgate, 2006)]. The paradigm of asthma as a $T_H2$ disorder is challenged by the discovery of elevated levels of $T_H1$ cytokines such as IFN-γ and IL-12 in some cases of human asthma[(Kenyon *et al.*, 2000; Brown *et al.*, 2003)]. In contrast to the mouse studies described above, clinical trials targeting $T_H2$ cytokines were disappointing[(Borish *et al.*, 1999; Leckie *et al.*, 2000)].

Not only has the $T_H2$ nature of asthma been challenged, so has the notion that it is mediated purely by the adaptive immune system[(Akbari *et al.*, 2003; Kim *et al.*, 2008; Hammad *et al.*, 2009)]. It has become apparent that asthma is far more complex, perhaps a syndrome (i.e. a defined collection of symptoms) rather than a disease with a specific cause such as atopy or allergy[(Pearce *et al.*, 1999; Borish and Culp, 2008)].

In human asthma, the smooth muscle cells of the airways undergo hyperplasia and/or hypertrophy, often described as 'remodelling'[(James and Carroll, 2000)]. This phenomenon is central to true human asthma and is the cause of airway hyperresponsiveness[(Zuyderduyn *et al.*, 2008)].

Mouse models of asthma have been developed or advanced to take into account airway hyperresponsiveness[(Henderson *et al.*, 2002)]. However, even in more developed models, the cause of hyperresponsiveness may be allergy/thickening of mucosa, not the constriction of smooth muscle[(Wagers *et al.*, 2004)]. Even those models which do involve remodelling of the airway tissues are limited: Remodelling involves deposition of extracellular matrix material, fibrosis and inflammation of the pulmonary vasculature; phenomena that do not occur in human asthma[(Tormanen *et al.*, 2005; Xisto *et al.*, 2005)]. Interestingly, however, there is evidence that some earlier mouse models of allergic asthma do induce the smooth muscle changes associated with hyperresponsiveness[(Koya *et al.*, 2006; Southam *et al.*, 2007)]. Nevertheless, even in more representative models, the actual measurement of hyperresponsiveness in mice is problematic and controversial[(Adler *et al.*, 2004; Bates *et al.*, 2004)].

Human asthma is likely the result of chronic exposure to allergen and/or other triggers whereas animal models generally involve acute doses. Attempts to bring the induction of allergic asthma model more in line with that of true human asthma actually led to tolerisation[(Yiamouyiannis *et al.*, 1999)]. In order to make models of allergic asthma more relevant to the human disease, known allergens such as extracts of dust mite and ragweed were coadministered. These exacerbate disease and are able to replace the traditional, and very artificial, alum adjuvant[(Fattouh *et al.*, 2005; Goplen *et al.*, 2009)]. In addition, these studies were able to avoid the tolerisation described above.

Finally, non-allergic induction of airway hyperresponsiveness using cationic proteins directly targeting smooth muscle, either alone or in combination with allergic challenge, may provide models more relevant to human asthma[(Homma *et al.*, 2005; Bates *et al.*, 2008; Allen *et al.*, 2009)].

Asthma is a complex condition affecting humans only. It is therefore likely that all models will be significantly limited.

### 7.1.2. Mast cells and IgE in asthma

Mast cells reside in the peripheral tissues and express the high-affinity IgE receptor FcεRI. This receptor is also found on basophils and, in humans, on many other cell types including eosinphils and APCs[Kinet, 1999]. Mainly due to this receptor the majority of IgE, unlike other immunoglobulin molecules, is cell-bound rather than free in the plasma. Crosslinking of IgE molecules by antigen triggers degranulation of mast cells and basophils, causing the classical signs of the early phase of the allergic reaction[Wedemeyer *et al.*, 2000]. In addition, mast cells secrete IL-4 – this is the initial $T_H2$ stimulant, recruiting T cell help[Sherman *et al.*, 1999; Wang *et al.*, 1999b]. In this way IgE confers on the mast cell antigen-specificity, allowing it to effectively act as a coordinator between the innate and acquired immune responses.

The IgE-mast cell system has the potential for at least two positive feedback loops: Firstly, increased levels of IgE increases the number of FcεRI molecules on the surface of mast cells, increasing their sensitivity to antigen-induced crosslinking[Yamaguchi *et al.*, 1997; Smurthwaite *et al.*, 2001; Macglashan, 2005]. Secondly, activated mast cells can induce localised B cells to undergo class-switching to IgE via expression of cytokines (IL-4 and IL-13) and costimulatory molecules (CD40L)[Gauchat *et al.*, 1993; Coker *et al.*, 2003; Takhar *et al.*, 2005]. This positive-feedback system, and the opportunity to break it, has made IgE a key target for treatment of atopic conditions[Chang, 2000] and may also explain why IgE expression has evolved to become tightly regulated[Oettgen and Geha, 1999; Hellman, 2007].

Differentiation of smooth muscle cell bundles into a more contractile phenotype is a key feature of asthma, being the primary cause of airway restriction. Mast cell progenitors are recruited to inflamed airways by CXCR2-regulated VCAM-1[Hallgren *et al.*, 2007]. Infiltration of mast cells into airway smooth muscle defines the difference between human airway hyperresponsiveness associated with asthma and eosinophilic bronchitis, a disease closer to mouse models of allergic airway inflammation[Brightling *et al.*, 2002]. Several studies suggest that mast cell infiltration of airway smooth muscle is key in inducing this differentiation[Amin *et al.*, 2005; Woodman *et al.*, 2008], although recent work questions this hypothesis[Kaur *et al.*, 2010]. New mechanisms by which mast cells may be involved in the pathogenesis of asthma are still being described[Skokos *et al.*, 2003; Kambayashi *et al.*, 2008; Margulis *et al.*, 2009]. The role of mast cells in asthma is clearly complex and will not be exhaustively discussed in this work.

### 7.1.3. T cells in asthma

Mast cell activation also readies the local environment for the late-phase response. Increased vascular permeability and expression of TNF-α causes infiltration of leukocytes to the site of antigen. Mast cell-synthesised IL-4 and IL-13 can induce DCs to bias infiltrating cells towards a $T_H2$-skewed response[Webb *et al.*, 2007]. T-cell produced IL-4 is vital for their own migration; T cells transferred from IL-$4^{-/-}$ mice are not recruited to the lung[Cohn *et al.*, 1997]. Perhaps more important though is mast cell-derived TNF-α. Indeed, recruitment of T cells, even from IL-$4^{-/-}$ mice can be artificially induced by local administration of TNF-α[Wershil *et al.*, 1991; Cohn *et al.*, 1997]. T cell help enhances the $T_H2$ bias by expression of IL-4, IL-13 and CD40L as well as inducing eosinphilia by production of IL-5 (see 7.1.4).

### 7.1.4. Eosinophils in asthma

Whilst mast cells may be regarded as the cells involved in the initiation of an episode of asthma, eosinophil infiltration can be considered its defining histologic feature[(Wardlaw *et al.*, 1995)]. Clinical studies show a correlation between eosinophil numbers and severity of disease[(Bousquet *et al.*, 1990; Rao *et al.*, 1996; Duncan *et al.*, 2003)]. The potential of eosinphils to secrete effector molecules that could directly cause the characteristic symptoms of asthma is consistent with the notion that eosinophils are the final-stage effectors of asthma[(Thomas and Warner, 1996)].

The primary chemoattractant for eosinophils is eotaxin, produced by fibroblasts, endothelial cells and epithelial cells during inflammation[(Garcia-Zepeda *et al.*, 1996)]. TNF-α, IL-4 and IL-13, produced by infiltrating $T_H2$ cells as well as mast cells, enhance eotaxin expression[(Lilly *et al.*, 1997; Li *et al.*, 1999a)]. The $T_H2$ nature of eosinphilia is demonstrated by the inbibition of eotaxin expression by the $T_H1$ cytokines IFN-γ and IL-12[(Miyamasu *et al.*, 1999; Zhao *et al.*, 2000)].

The cytokine with the most obvious relationship with eosinophilia is IL-5. IL-5 may increase eosinophil numbers by enhancing emigration from the bone marrow[(Palframan *et al.*, 1998)] or by increasing eosinophil longevity[(Simon *et al.*, 1997)]. Whatever the precise mechanism, in the absence of IL-5, either through genetic knockout[(Foster *et al.*, 1996)] or by administration of passive antibody[(Coffman *et al.*, 1989; Van Oosterhout *et al.*, 1993; Kurup *et al.*, 1997; Stein *et al.*, 2008)], eosinophil infiltration and activation are dramatically reduced. Therefore, whilst eosinophilia is the key effector of disease symptoms, $CD4^+$ T cells, as IL-5-producers, are responsible for recruiting eosinophils and are viable targets for therapy.

Finally, it is becoming understood that eosinophils have significant antigen-presenting and lymphocyte-recruitment capability during airway inflammation[(Wang *et al.*, 2007; Jacobsen *et al.*, 2008; Spencer *et al.*, 2009)]. This eosinphil:lymphocyte feedback loop, along with the mast cell:IgE:B cell feedback loop described above, are key factors in the induction and perpetuation of asthma and are likely to contribute significantly to the difficulty in treating the disease.

### 7.1.5. Basophils in asthma

Basophils are mature granulocytes and are recruited from the circulation to peripheral tissues by allergic inflammation or helminth infection.

Recently, they have been associated with the development of $T_H2$ responses[(Sokol *et al.*, 2008)]. Genetically modified mice with a $T_H2$ polarisation have a highly expanded basophil population and splenocytes from these mice, producing IL-4, can induce naïve T cells to express IL-4 in response to antigen *in vitro*[(Hida *et al.*, 2005)]. Similarly, artificially increasing basophil numbers *in vivo* by administration of IL-3 enhances, in an IL-4-dependent manner, the $T_H2$ response to antigen[(Oh *et al.*, 2007)]. There is also evidence that basophils can act independently as antigen presenting cells[(Sokol *et al.*, 2009)].

Increased airway basophil frequency and activation level has been described in asthmatics, in particular during incidents of asthma[(Macfarlane *et al.*, 2000; Kepley *et al.*, 2001; Schroeder *et al.*, 2001; Yoshimura *et al.*, 2002)]. Conversely, successful treatment of asthma with anti-IgE is associated with reduced basophil FcεRI expression and, more importantly, degranulation[(MacGlashan *et al.*, 1997; Lin *et al.*, 2004a)].

### 7.1.6. Existing treatments

The major treatment for asthma is currently steroid drugs, either alone or in combination with beta 2 agonists. This treatment has been effective in general but a significant proportion of patients do not respond to treatment. Those that do are normally subject to long-term, often lifelong, treatment which is unpopular and can have side-effects. Recently, the involvement in regulatory T cells in the therapeutic effect of steroid therapy has been identified. Deficiency in number and/or activity of regulatory T cells in asthmatic lungs have been restored by corticosteroid treatment(John *et al.*, 1998; Karagiannidis *et al.*, 2004; Hartl *et al.*, 2007; Ali *et al.*, 2008). Coadministration of 1,25-Dihydroxyvitamin D3 has been shown to enhance this effect on regulatory T cells(Barrat *et al.*, 2002; Topilski *et al.*, 2004; Bartels *et al.*, 2007). Interestingly, therefore, the mechanism of action of traditionally broad-acting, antigen nonspecific steroids is at least partly carried out by regulatory T cells.

Aside from steroid-based therapies, immunosuppressants, specifically calcineurin inhibitors, previously restricted to anti-rejection therapy, have shown considerable promise in treatment of atopic disease when administered either locally(Hultsch *et al.*, 2005) or systemically(Madan and Griffiths, 2007). These drugs inhibit T cell activity, therefore breaking the positive-feedback cycle described above. Although they may avoid many of the long-term side effects of steroid treatment, they remain just as antigen-nonspecific. This therefore allows an, at least theoretical, possibility of increased risk of unchecked infection and neoplasia.

Specific immunotherapy (SIT), discussed in sections 1.4 and 1.5, has shown some success in allergy-induced asthma (Penagos *et al.*, 2008)(reviewed in(Mohapatra *et al.*)). Refinement of treatment is ongoing but exposure of allergic individuals to allergen carries with it obvious risks. Similarly to steroid therapy, this therapy requires long-term treatment.

As mentioned in 0, IgE has become a target for asthma therapy. The humanised monoclonal $IgG_1$, omalizumab, has been successfully introduced as a treatment for asthma(Chang and Shiung, 2006). In developed countries, modern hygeine has dramatically reduced our exposure to the classical targets of IgE, helminths. Therefore, despite its antigen-nonspecificity, the theoretical risks of a systemic reduction of IgE are perhaps lower than those of nonspecific T-cell inhibitors.

As noted in 7.1.4, T cells are viable targets for anti-allergy therapy. In contrast to the above treatments, the targeting of specific T cells potentially allows for antigen-specific, curative, treatment of disease. The existing antigen-specific treatment for allergic disease is known as specific immunotherapy (SIT)(Larche *et al.*, 2006) (discussed in detail in the introduction to this thesis). A wide range of protocols exist but, in general, they involve extended and/or noninflammatory exposure to antigen. IL-10 appears to be intrinsically involved in the effect of SIT although the mechanism is still unclear(Akdis *et al.*, 1998; Hawrylowicz *et al.*, 2002; Xystrakis *et al.*, 2006). In addition, it may be possible that, because of its reliance on the natural response to antigen, SIT may be ineffective in cases where a residual pathological response to a previous exposure still endures(Hurst *et al.*, 2001). Tolerisation via DNA immunisation should allow the introduction of relevant immunomodulatory molecules in order to produce similar results to successful SIT but more consistently and via a more convenient procedure.

### 7.1.7. IL-10 vs asthma

Impaired IL-10 production has been suggested as a potential cause of asthma[(John *et al.*, 1998; Matsumoto *et al.*, 2004)]. Studies in knockout mice agree[(Tournoy *et al.*, 2000)]. *In vitro*, corticosteroids have been shown to promote IL-10 synthesis[(Hodge *et al.*, 1999; Richards *et al.*, 2000)]. In agreement, effective corticosteroid treatment is associated with increased IL-10 production whilst nonresponsiveness to steroids correlates to a poor IL-10 response[(John *et al.*, 1998; Hawrylowicz *et al.*, 2002; Karagiannidis *et al.*, 2004; Xystrakis *et al.*, 2006)]. As discussed in the introduction chapter, IL-10 is able to induce regulatory T cells via several mechanisms. It is therefore a valid candidate molecule for the treatment of allergic asthma. Perhaps just as relevant to asthma, IL-10 is also able to downregulate IgE receptor expression on mast cells[(Kennedy Norton *et al.*, 2008)] and dendritic cells[(Faith *et al.*, 2009)]. Previous work has shown that intranasal administration of IL-10[(Zuany-Amorim *et al.*, 1995)] or IL-10-encoding viral vector[(Stampfli *et al.*, 1999)], intravenous administration of IL-10-encoding plasmid[(Nakagome *et al.*, 2005)] and intratracheal administration of IL-10-expressing DC[(Henry *et al.*, 2008)] are able to inhibit disease in ova-induced asthma models.

As mentioned earlier, IL-10 has been described both as a $T_H2$ and as a $T_R1$ cytokine. It may well be that in a $T_H1$-based immunological disease, the distinction is less important; a $T_H2$ influence might sufficiently balance the response and alleviate symptoms. However, in a $T_H2$ disease such as the allergic asthma model in this study, coadministration of antigen and IL-10 genes must promote a regulatory response in order to abrogate disease. Indeed, if IL-10 were ineffective in doing so, it may be more effective to rely on the intrinsic $T_H1$-inducing nature of DNA vaccines and to immunise with antigen gene alone[(Hsu *et al.*, 1996; Raz *et al.*, 1996; Zindler *et al.*, 2008)]. However, it remains likely that the induction of $T_R1$ tolerance is a more rational approach than is $T_H1$ shifting[(Tournoy *et al.*, 2002)].

### 7.1.8. Aim of experiments

This chapter examines the effect of prophylactic DNA immunisation on the induction of disease. Specifically, it asks if IL-10 is sufficient, when present at the site of antigen presentation, to inhibit a future asthma-inducing antigenic challenge.

## 7.2. Results

The original antigen-induced asthma model used in this chapter was developed at GlaxoSmithKline. Briefly, $1x10^6$ DO11.10 cells are adoptively transferred into naïve Balb/c mice. The recipient mice are sensitised by intraperitoneal (i.p.) administration of ovalbumin in alum. One week later asthma is induced by intranasal (i.n.) administration of ovalbumin solution. The asthma is characterised by increased airway eosinophilia, a $T_H2$ shift of serum antibody and production of $T_H2$-associated cytokines such as IL-4. This chapter examines the effect of PMDD immunisation on the outcome of asthma induction by introducing a prime-boost immunisation before the initial sensitisation step. This model is illustrated in more detail in Figure 7-1. It should be noted that the names of test groups are


| | **"Adoptive transfer"** 1x10$^6$ DO11.10 cells into Balb/c recipients | **"Prime"** PMDD immunisation | **"Boost"** PMDD immunisation | **"Sensitisation"** i.p. OVA-alum | **"Asthma induction"** i.n. solution | **"Analysis"** BAL, lymph nodes, blood obtained. |
|---|---|---|---|---|---|---|
| "Control" | ✓ | p7 | p7 | ✓ | PBS | ✓ |
| "Naïve" | ✓ | p7 | p7 | ✓ | OVA-PBS | ✓ |
| "Primed" | ✓ | p7.OVA | p7.OVA | ✓ | OVA-PBS | ✓ |
| "Treated" | ✓ | p7.OVA + p7.IL10 | p7.OVA + p7.IL10 | ✓ | OVA-PBS | ✓ |

**Figure 7-1. The asthma model**
The original asthma model consisted of adoptive transfer, sensitisation, induction and analysis. The model used in this chapter incorporates a prime-boost strategy as seen in earlier chapters. 24 h following adoptive transfer, mice were immunised (”prime”) with empty p7 (”control” and “naive”), p7.OVA (”primed”) or p7.OVA+p7.IL10 (”treated”). Two weeks later mice received a second immunisation (”boost”) with the same vector(s). 14 days post-boost mice were sensitised by intraperitoneal administration of 10 µg OVA adsorbed to 2 mg alum in 0.2 ml sterile saline. 10 days post-sensitisation asthma was induced by intranasal administration of 50 µg OVA in 50 µl saline (”naive”, “primed” and “treated” groups); repeated on three consecutive days. The “control” group instead received intranasal saline. 3 days post-induction, mice were sacrificed and bronchoalveolar lavage (BAL) fluid, lymph nodes and blood were obtained.

imperfect descriptions. For example the "naïve" group is naïve only with regards to the PMDD immunisations, before the OVA-alum sensitisation step.

Mice underwent a prime-boost procedure with either empty p7, p7.OVA or p7.OVA + p7.IL10. As previously, the first immunisation (prime) took place one day following adoptive transfer and the second (boost) four weeks after that. Two weeks post-boost the asthma model was resumed: Mice were sensitised and asthma induced (henceforth refered to as sensitisation/induction) as described above.

### 7.2.1. Effect of DNA immunisation on IgE responses

IgE levels in blood plasma were measured by ELISA (Figure 7-2). A significant increase in OVA-specific IgE was observed in both groups that received p7.OVA; the coadministration of p7.IL10 had no observable effect on the concentration of OVA-specific IgE in the plasma (Figure 7-2a). In contrast, the level of total (nonspecific) IgE was further increased by coadministration of p7.IL10 (Figure 7-2b).

### 7.2.2. Effect of DNA immunisation on IgG responses

The $T_H1/T_H2$ balance of an immune response, or of the state of the immune system in general, is often, at least in part, observed via the relative prevalence of IgG subtypes. In particular, elevated levels of $IgG_2$ indicate a shift towards a cellular, $T_H1$, response[(Kuperman *et al.*, 1998; Randolph *et al.*, 1999b)].

OVA-specific IgG subtypes were quantified by ELISA (Figure 7-3). A significant increase in $IgG_1$ was observed after sensitisation/induction ('naïve' group) but no further increase was induced by pre-immunisation with p7.OVA. Coadministration of p.IL10 also had no observable effect on $IgG_1$.

Similarly to $IgG_1$, $IgG_{2a}$ expression was increased by sensitisation/induction. This was increased significantly by pre-immunisation with p7.OVA. Mice that received coadministered p7.IL10 exhibited significantly lower plasma concentrations of $IgG_{2a}$ compared to those that received p7.OVA alone. However the concentration remained significantly higher than mice that received empty p7 alone.

A similar pattern was observed in $IgG_{2b}$ concentrations but with key differences: Firstly, sensitisation/induction alone was not sufficient to elevate levels above those in control mice. Secondly, although immunisation with p7.OVA significantly increased $IgG_{2b}$ plasma concentration, coadministration of p7.IL10 was effective in completely eliminating this. Taken together, these data suggest that the quantitative activation threshold for class-switching to $IgG_{2b}$ is higher than that for $IgG_{2a}$.

$IgG_3$ plasma levels exhibited a similar pattern to those of $IgG_1$: Unprimed sensitisation/induction caused a significant increase in $IgG_3$ compared to control mice but neither p7.OVA alone nor p7.OVA+p7.IL10 had any further effect. This is not necessarily unexpected; class switching to $IgG_3$ appears to require activation of TLR4[(Quintana *et al.*, 2008)]. Although this might conceivably be achieved through the release of heat-shock proteins by cell damage caused by the particle bombardment, the more classical TLR4 ligand, bacterial-derived LPS, is not present.

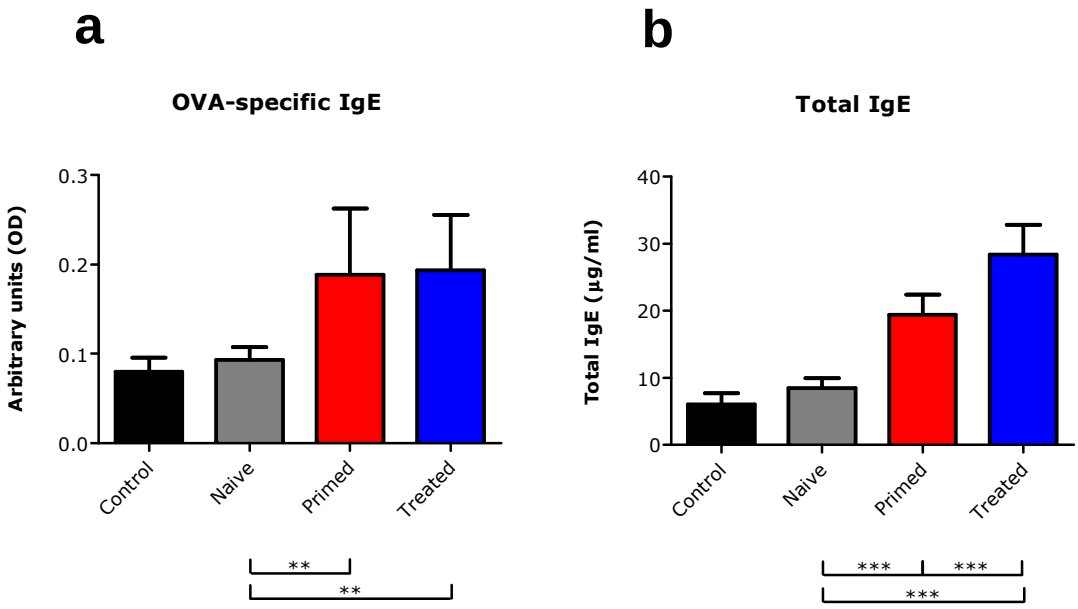



**Figure 7-2. Prophylactic p7.IL10 does not inhibit a p7.OVA-induced increase in serum IgE following induction of asthma.**

Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised ('prime') with either empty p7 (naive), p7.OVA (primed) or p7.OVA + p7.IL10 (treated). Four weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were sensitised to ovalbumin by intraperitoneal administration in an alum adjuvant. One week after sensitisation asthma was induced by intranasal administration of ovalbumin solution. A control group received prime and boost immunisations of empty p7, sensitisation with IP ova-alum and índuction with intranasal PBS. 84 h post-induction all mice were sacrificed and blood was obtained by severing the aorta.

OVA-specific (a) and total (b) IgE were measured by ELISA. Because no standard OVA-specific IgE was available, quantification was not possible, therefore OVA-specific IgE data are displayed in arbitrary units (optical density).

Data are the means of three independent experiments.

***p<0.001; **p<0.01 by one-way ANOVA

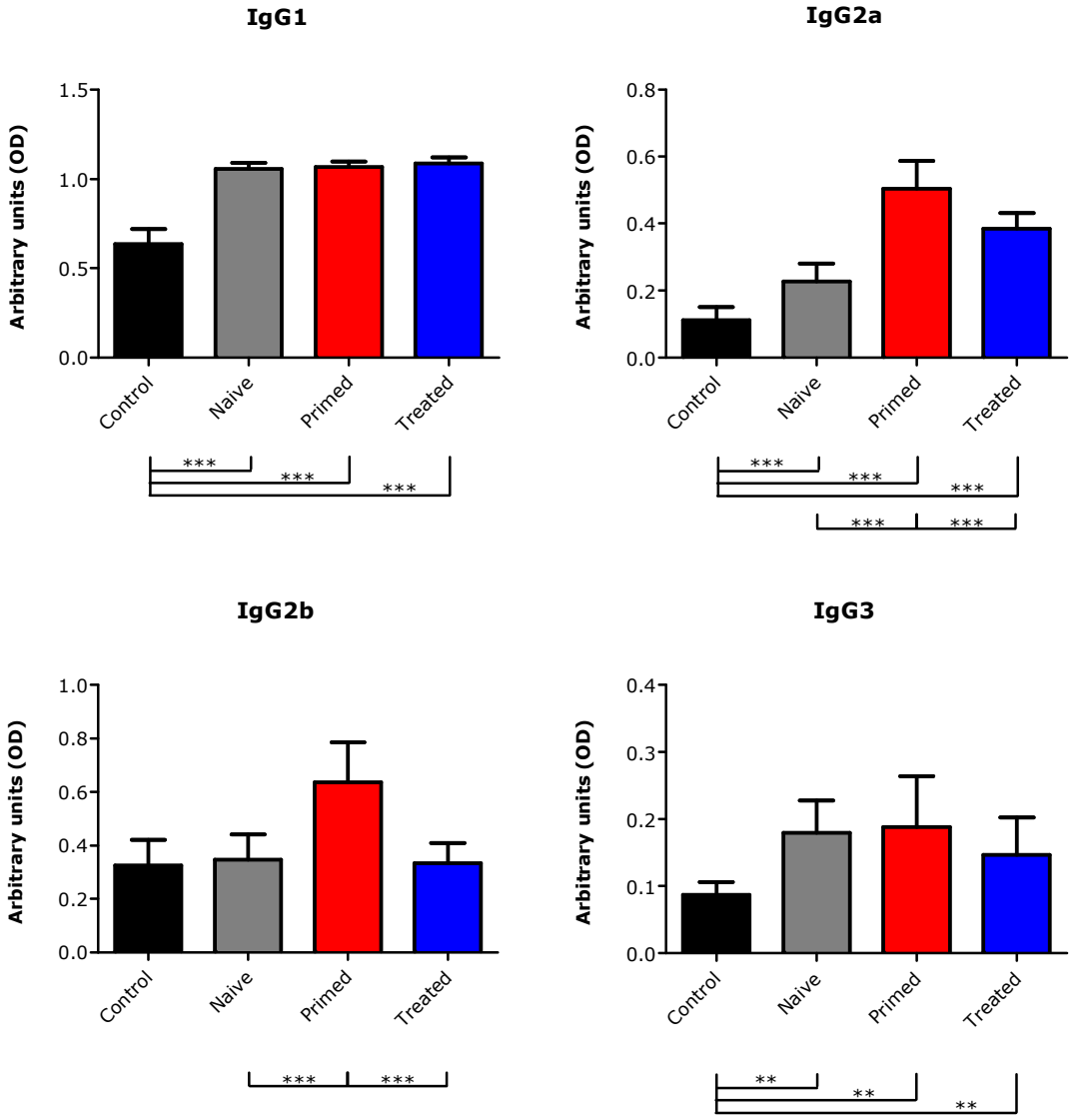



**Figure 7-3. Prophylactic p7.IL10 inhibits a p7.OVA-induced increase in serum $T_H1$ IgG subtypes following induction of asthma.**

Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised ('prime') with either empty p7 (naive), p7.OVA (primed) or p7.OVA + p7.IL10 (treated). Four weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were sensitised to ovalbumin by intraperitoneal administration in an alum adjuvant. One week after sensitisation asthma was induced by intranasal administration of ovalbumin solution. A control group received prime and boost immunisations of empty p7, sensitisation with IP ova-alum and índuction with intranasal PBS. 84 h post-induction all mice were sacrificed and blood was obtained by severing the aorta.

OVA-specific IgG subtypes were measured by ELISA. Because no standard OVA-specific IgG was available, quantification was not possible, therefore data are displayed in arbitrary units (optical density).

Data are the means of three independent experiments. Repeated-measures ANOVA was performed on data across serial dilutions.

**p<0.01; ***p<0.001

### 7.2.3. LN ELISpot

To examine the cytokine response to sensitisation/induction mediastinal lymph nodes were harvested 84 h following intranasal challenge. OVA-dependent cytokine expression by lymph node (LN) cells was examined by ELISpot (Figure 7-4).

Compared to control mice, those that were naïve until sensitisation/induction exhibited significantly increased numbers of IL-2-expressing cells (Figure 7-4a). Earlier prime-boost with p7.OVA did not increase this number further. However, coadministration of p7.IL10 was able to reduce the prevalence of IL-2-producing cells by a small but significant number.

Unprimed sensitisation/induction was also able to increase the number of IL-4-expressing LN cells compared to the number in control mice (Figure 7-4b). Unlike IL-2, however, the number of cells producing IL-4 in response to OVA was increased futher by prime-boost with p7.OVA. Interestingly, coadministration of p7.IL10 caused a statistically insignificant, but noticeable, increase in the prevalence of IL-4-producing cells. This indicated that immunisation with p7.OVA alone was able to prime for the IL-4 response induced by OVA-alum and that this effect was not diminished by coadministration of p7.IL10.

### 7.2.4. BAL ELISpot

To better understand the immune response at the site of inflammation, bronchoalveolar lavage (BAL) was performed on mice at the time of sacrifice. Attempts to measure cytokine levels in the BAL fluid failed but examination of cells in the fluid was successful.

ELISpot was performed on BAL cells (Figure 7-5). Control mice had fewer than 100 IL-2-producing cells per million BAL cells (Figure 7-5a). Sensitisation/induction increased this number significantly. Similar to what was observed in LN cells, prime-boost with p7.OVA did not further increase the number of IL-2-producing cells. Interestingly, prior immunisation with p7.OVA+p7.IL10 did increase the number of IL-2-producing cells – by an order of magnitude compared to both naïve and p7.OVA-treated mice. This was in contrast to the lack of effect of p7.I10 in the lymph node IL-2 ELISpot.

When examining IL-4 expression in BAL cells it was observed that background expression, in the absence of exogenous $OVA_{323-339}$, was far higher than normal (Figure 7-5b). Ordinarily, background wells have fewer than 10% of the number of spots as do test wells and the background figure is subtracted from the test figure. In this test, the numbers were almost equal in both groups that received p7.OVA (either with or without coadministered p7.IL10). This suggests that cells in the BAL fluid spontaneously produce IL-4 without the need for further *in vitro* stimulation. To present this data clearly, rather than subtracting them, background values are displayed alongside test values.

Again, sensitisation/induction produced a significant increase in cytokine-expressing cells compared to the control group. Unlike IL-2-producers, however, IL-4-producers were three times more prevalent following p7.OVA priming. A further significant increase was provided by coadministration of p7.IL10.

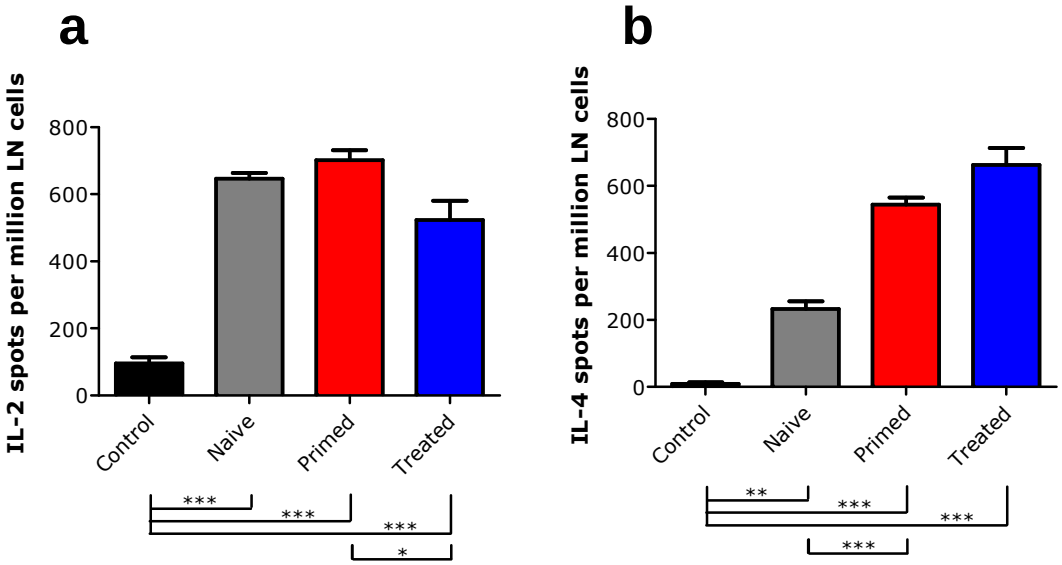



**Figure 7-4. Prophylactic p7.IL10 reduces the number of IL-2-, but not IL-4-, producing OVA-specific lymphocytes in the draining lymph nodes following induction of asthma.**

Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised ('prime') with either empty p7 (naive), p7.OVA (primed) or p7.OVA + p7.IL10 (treated). Four weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were sensitised to ovalbumin by intraperitoneal administration in an alum adjuvant. One week after sensitisation asthma was induced by intranasal administration of ovalbumin solution. A control group received prime and boost immunisations of empty p7, sensitisation with IP ova-alum and índuction with intranasal PBS. 84 h post-induction all mice were sacrificed and cell populations from mediastinal lymph nodes were examined for OVA-dependent IL-2 and IL-4 expression by ELISpot.

Data are representative of three repeated experiments.

*** $p<0.001$; ** $p<0.01$; * $p<0.05$ by one-way ANOVA

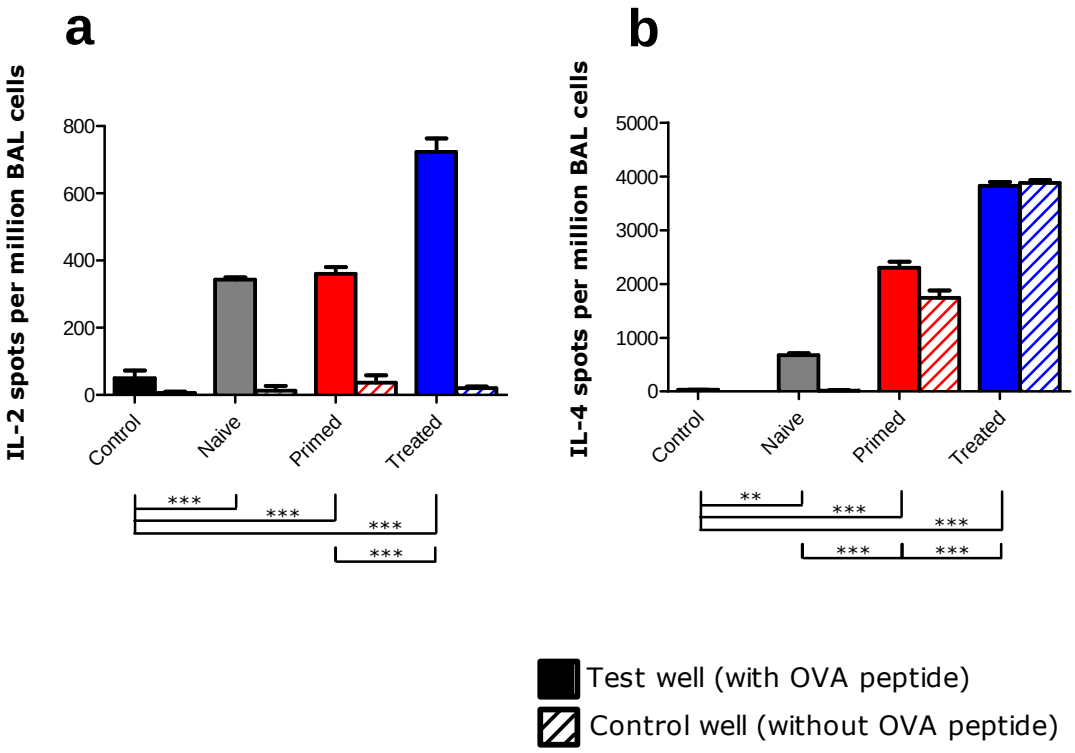



**Figure 7-5. Prophylactic p7.IL10 increases the number of IL-2- and IL-4-producing OVA-specific lymphocytes in the BAL fluid following induction of asthma.**

Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised ('prime') with either empty p7 (naive), p7.OVA (primed) or p7.OVA + p7.IL10 (treated). Four weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were sensitised to ovalbumin by intraperitoneal administration in an alum adjuvant. One week after sensitisation asthma was induced by intranasal administration of ovalbumin solution. A control group received prime and boost immunisations of empty p7, sensitisation with IP ova-alum and índuction with intranasal PBS. 84 h post-induction all mice were sacrificed and cell populations from bronchoalveolar lavage (BAL) fluid were examined for OVA-dependent IL-2 and IL-4 expression by ELISpot.

Unlike all other ELISpot data where background spots (from wells without exogenous $OVA_{323-339}$) are subtracted from test values (from wells with exogenous $OVA_{323-339}$) to provide a net, antigen-induced, figure, these graphs display both test (solid bars) and background (striped bars) figures. Data are representative of three repeated experiments.

*** $p<0.001$; ** $p<0.01$ by one-way ANOVA

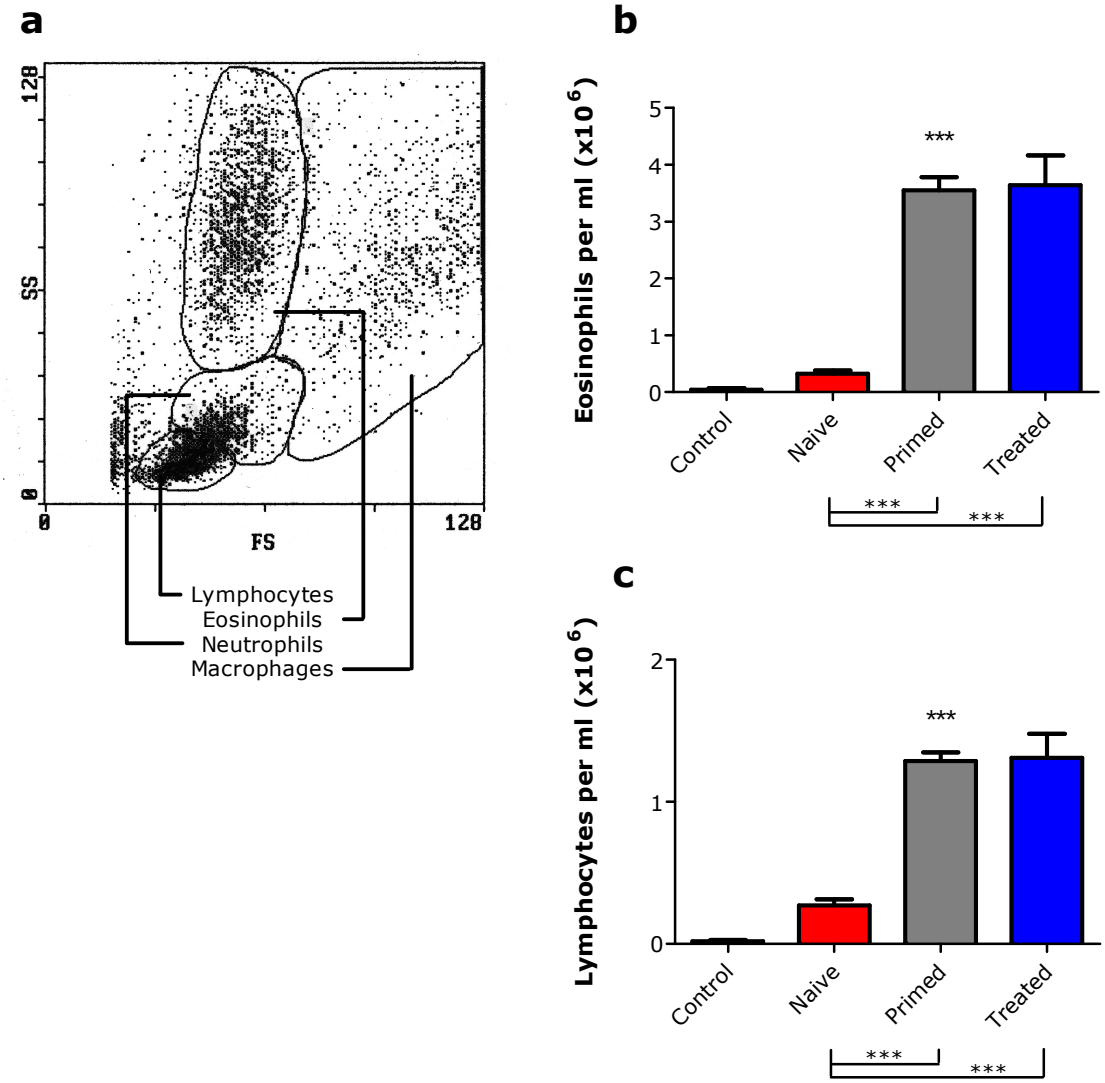



**Figure 7-6. The inclusion of p7.IL10 in prophylactic immunisation has no effect on the p7.OVA-induced increase in cellular infiltration to the lung following induction of asthma.**

Balb/c mice (3 per group) were adoptively transferred with $1x10^6$ DO11.10 T cells. 1 day later they were immunised ('prime') with either empty p7 (naive), p7.OVA (primed) or p7.OVA + p7.IL10 (treated). Four weeks later mice were re-immunised with the same vector ('boost'). Two weeks post-boost mice were sensitised to ovalbumin by intraperitoneal administration in an alum adjuvant. One week after sensitisation asthma was induced by intranasal administration of ovalbumin solution. A control group received prime and boost immunisations of empty p7, sensitisation with IP ova-alum and índuction with intranasal PBS. 84 h post-induction all mice were sacrificed and immune cell populations in the bronchoalveolar lavage (BAL) fluid were counted using forward- and side-scatter (FSC and SSC) flow cytometry.
(a) FSC-SSC plot with gates for four key cell populations. (b) Count of eosinophils per ml of BAL fluid. (c) Count of lymphocytes per ml of BAL fluid. Figures are the mean of three identical experiments.
*** $p<0.001$ by one-way ANOVA

### 7.2.5. Effect of DNA immunisation on BAL cell subsets

As noted above, a key factor in the pathology of asthma is the infiltration into the airway of inflammatory cells and their activation. These cell populations were examined by performing flow cytometry on the BAL fluid of mice at the time of sacrifice (Figure 7-6).

Cell populations were gated according size (forward-scatter; fs) and granularity (side-scatter; ss) (Figure 7-6a). Gates were previously defined by reverse-plotting of cell markers onto the fs/ss plot (data kindly provided by Maria Daly, GSK). BAL fluid from control mice contained fewer than $1x10^5$ eosinophils per ml and that from sensitised/induced mice contained up to $5x10^5$. Previous immunisation with p7.OVA significantly increased the number of eosinophils in BAL fluid; by approximately an order of magnitude. Coadministration of p7.IL10 had no effect on this infiltration. An almost identical pattern was seen in the lymphocyte population of the BAL fluid but on a smaller scale (Figure 7-6b).

## 7.3. Discussion

The data generated by the experiments in this chapter are summarised inTable 7-1. As evidenced by levels of IL-4 production and eosinophil infiltration prior immunisations with p7.OVA increases disease severity in this model. These data also show that coadministration of p7.IL10 does not impede this exacerbatory effect; much less protect against disease induction. This agrees with data in earlier chapters which show that p7.IL10 can inhibit an immediate response to p7.OVA but does not induce tolerance or protect against a later challenge.

### 7.3.1. Immunisation with p7.OVA selectively enhances serum antibody isotype levels whilst inclusion of p7.IL10 promotes a $T_H2$ antibody isotype bias.

Allergic disorders such as asthma and allergic rhinitis are often associated with elevated plasma levels of IgE. This despite the fact that, unlike the majority of antibodies, most IgE is cell-associated *before* binding antigen[(Metzger, 1992; Bonnefoy *et al.*, 1993)]. In agreement with this, the serum concentration of both OVA-specific and nonspecific IgE was increased by prior immunisation with p7.OVA. In contrast, serum $IgG_{2a}$ and $IgG_{2b}$ concentrations were also increased; this normally implies a $T_H1$-biased response, especially when compared with the absence of such an increase in $IgG_1$.

Whilst coexistence of $T_H1$ and $T_H2$ responses to allergen have been described[(Li *et al.*, 1998; Hansen *et al.*, 1999; Randolph *et al.*, 1999a; Randolph *et al.*, 1999b)] it may also be possible that the asthma-inducing sensitisation-induction protocol increases serum $IgG_1$ to maximal levels and that further stimulation is unable to increase the concentration further. This would artificially distort the data, implying a $T_H1$-shift when, in fact, IgG subtype ratios might remain the same.

Nevertheless, the data in 7.2.1 clearly demonstrate increased serum concentrations of both OVA-specific and total IgE in mice in which asthma was induced. 0. This agrees with the literature on both experimental and clinical asthma (see 1.1.2).

| | Serum IgE ELISA | | Lymph node ELISpot | | BAL ELISpot | | BAL cell subtypes | |
|---|---|---|---|---|---|---|---|---|
| | **OVA-specific** (OD) | **Total** (µg) | **IL-2** (spots per million cells) | **IL-4** | **IL-2** (spots per million cells) | **IL-4** | **Eosinophils** (cells/ml) | **Lymphocytes** (cells/ml) |
| Control | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| Naïve | = | = | +++ | + | ++ | + | = | = |
| Primed | ++ | ++ | +++ | +++ | ++ | ++ | +++ | +++ |
| Treated | ++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ |

**Table 7-1. Summary of data from asthma experiments**
Relative changes in measured values as compared to control group.

The effect of inclusion of p7.IL10 in the immunisation is to further increase serum total IgE whilst causing a small but significant decrease in OVA-specific $IgG_{2a}$ and $IgG_{2b}$. Unfortunately the data do not provide an explanation for the unexpected observation that this treatment increases total, but not OVA-specific, IgE. However there is evidence elsewhere that the ratio of specific:nonspecific IgE is potentially much higher locally[(Smurthwaite *et al.*, 2001)]. In addition, there is a possibility that the continuous presence of antigen allows epitope-spreading via CD23-mediated Facilitated Antigen Presentation (FAP) by OVA-nonspecific B cells[(Mudde *et al.*, 1990; Karagiannis *et al.*, 2001; Carlsson *et al.*, 2007)]: Via CD23, B cells are able to internalise, process and present antigen which their BCR does not recognise if the antigen is bound by IgE. If the B cell is in an activated state and/or part of an antigen-presenting cluster this may provide sufficient stimuli to activate T cells which are specific to irrelevant antigen.

A caveat when examining IgE plasma data is that it has the potential to be unrepresentative of local IgE concentrations: A high local concentration of antigen and Fcε receptors has the potential to mop up locally-secreted IgE and, in addition, there is evidence that once local FcεRI receptors are saturated excess locally-produced IgE is preferentially secreted into mucous rather than blood[(Negrao-Correa *et al.*, 1996; Wilson *et al.*, 2002)]. Therefore the relationship between local and plasma IgE concentrations is unlikely to be linear. Nevertheless it is likely that an increase in plasma IgE would reflect increased local production.

IL-10 has been shown to block signalling through the ICOS costimulatory receptor[(Taylor *et al.*, 2007)] which, in turn is key in antibody class-switching to IgE[(McAdam *et al.*, 2001)]. It is therefore surprising that, in this study, the inclusion of IL-10 in the immunisation was unable to diminish the IgE response. One explanation for this could be the kinetics and location of the response: IL-10 blockade of ICOS through SHP-1-mediated dephosphorylation of the ICOS cytoplasmic tail is brief[(Taylor *et al.*, 2007)]. IL-10 provided by the immunisation would be localised to the abdominal skin and, if dendritic cells were directly transfected, the skin-draining lymph nodes. In contrast, class-switching to IgE tends to occur locally in the target organ[(Coker *et al.*, 2003; Takhar *et al.*, 2005)]. Transfected cells are unlikely to reach the lung or lung-draining lymph nodes. Rather, they would be required to exert their effect on naïve T cells which would subsequently encounter antigen elsewhere. If naïve T cells were insufficiently tolerised, either in quality or quantity, the generation of IgE-secreting plasma cells would remain possible.

### 7.3.2. Immunisation with p7.OVA alone enhances IL-4, but not IL-2, production

It is perhaps surprising, given the previously established ability of p7.OVA to stimulate T cell proliferation and IL-2 production, that pre-immunisation with the vector did not increase the number of OVA-specific IL-2-producing T cells beyond the number induced by the asthma-induction protocol. In contrast, the number of IL-4-producers in both lymph node and BAL was significantly increased by gene gun immunisation. Whilst accompanying flow cytometry data would have provided confirmation, examination of the lymph node ELISpot data suggests that prior immunisation with p7.OVA results in all OVA-responsive T cells being dual-producers of IL-2 and IL-4; in effect, $T_H2$ cells.

### 7.3.3. Inclusion of p7.IL10 in the immunisation increases the number of cytokine-producing cells at the site of inflammation.

It was shown earlier (6.2.1) that a prime-boost immunisation with p7.OVA+p7.IL10 is able to reduce the number of l/n IL-2 ELISpots in response to subsequent challenge compared to mice that received p7.OVA alone. However, that treatment was unable to reduce the number of IL-2-producing cells in the lymph nodes to below that of naive mice. In contrast, the data in this chapter show that treatment was able to reduce, by a small but significant number, the number of IL-2-producing cells in the draining lymph nodes. A caveat to this comparison is that the 'naïve' group in the former experiment were truly experiencing antigen for the first time whereas, in the latter, the 'naïve' group experienced antigen during the i.p. priming. In addition, the sample time in the former was 72 h post-immunisation and, in the latter, 84 h. Figure 5-5 shows that this 12-hour window provides the opportunity for much cell division, especially in naïve cells. Nevertheless, in both cases the inclusion of p7.IL10 in the immunisation reduces the number of IL-2-producing cells in the draining lymph nodes by roughly one third.

In contrast to the lymph nodes, the number of IL-2-producing cells in the BAL is doubled by inclusion of p7.IL10 in the immunisation. A similar effect is seen on IL-4-producing cells although the number of IL-4-producing cells in the BAL is an order of magnitude higher than the number of IL-2 producers.

Examining the lymph node data alongside the BAL data suggests that the inclusion of p7.IL10 in the immunisation either induces activated T cells to migrate to the lung or causes additional activation or proliferation in the lung. As described earlier (1.3.2), IL-10 can induce DC to migrate preferentially to the periphery, as opposed to lymph tissue[(Dieu-Nosjean *et al.*, 2001; Takayama *et al.*, 2001a; Demangel *et al.*, 2002)]. The inclusion of p7.IL10 in the immunisation may, therefore, potentially increase the quantity of antigen presentation at the site of inflammation. This supports, to some degree, the possibility that the additional cytokine production is due to increased proliferation and/or activation in the lung. In partial opposition to this is data that suggests T cells that have migrated to lung are unable to divide[(Harris *et al.*, 2002)]. This would therefore limit the mechanism to either increased T cell migration or an increased state of activation.

A phenomenon that is not seen elsewhere in this study is the large number of IL-4 ELISpots seen in 'background' control wells containing no exogenous antigen. Three possibilities exist: 1) Residual antigen. Antigen was last administered 84 h prior to sacrifice and there is no equivalent IL-2 background. This makes it unlikely that residual antigen is responsible, although it has been shown elsewhere that intranasal antigen can persist for several days[(Byersdorfer and Chaplin, 2001)]. 2) IL-4 is produced by another cell type. Mast cells and eosinophils both constitutively express IL-4 mRNA and are therefore primed for fast production of the cytokine[(Gessner *et al.*, 2005)]. Mast cell production of IL-4 is linked to local IgE which, although not directly measured, is likely to be elevated. The cycle described in 0 provides a niche for long-term IgE production and, therefore, mast cell sensitisation[(Smurthwaite *et al.*, 2001; Hiepe and Radbruch, 2006; Radbruch *et al.*, 2006)]. It would have been enlightening to have performed IgE ELISA on BAL fluid to confirm local concentrations. Although mast cells were not enumerated by

morphologic flow cytometry, it is possible to calculate that, in the "primed" and "treated" groups, 50-60% of cells in a BAL ELISpot well would be eosinophils whilst only 20% are lymphocytes. This certainly allows for eosinophilic production of IL-4 to contribute significantly to the total number of IL-4 ELISpots. 3) The kinetics of T cell IL-4 production are different to those of IL-2 production. There is certainly evidence that IL-4 can be produced for an extended period of time post-stimulation[(Laaksonen *et al.*, 2003)]. Previous work by Prof. B.M. Chain has shown similarly high background IL-4 spots in the spleen following repeated immunisation of mice with sheep red blood cells (unpublished data).

Flow cytometry of intracellularly cytokine-stained cells would likely provide a clearer picture of the cell type(s) producing IL-4. Due to time constraints, however, this experiment could not be repeated further and, as yet, the cause remains unexplained.

### 7.3.4. The cellular infiltration caused by immunisation with p7.OVA is not reduced by coadministration of p7.IL10

Repeated immunisation with p7.OVA prior to asthma induction increased the number of lung-infiltrating lymphocytes by four times and the number of eosinophils by an order of magnitude. The coadministration of p7.IL10 had no effect on infiltration. Because immunisation was at a distal site priming of the airway specifically is unlikely and, therefore, the mechanism involved in this disease exacerbation must be systemic. The pattern of a p7.OVA-induced increase which is unaffected by p7.IL10 is similar to that of total plasma IgE. Increased OVA-specific IgE caused by immunisation would prime mast-cells systemically, allowing a swift and powerful response to antigenic challenge, wherever it may be. Intranasal challenge would activate local mast cells, which, in turn, would allow infiltration of lymphocytes and(, later,) eosinophils.

One might expect IL-4 production in the lung to follow a similar pattern to that of lymphocyte infiltration and OVA-specific IgE production. However the BAL ELISpot data show a further increase in mice that received coadministered p7.IL10. This could be due to the background IL-4 production as discussed in 7.3.3 or to the fact that all *in vivo* immunisations and challenges are performed with OVA protein whereas this ELISpot (and most of the others in this study) is performed with $OVA_{323-339}$ peptide. Section 1.2.2 previously demonstrated that a significant number of T cells are primed to non 323-339 epitopes of the OVA protein. It is also possible that differences in ELISpot data between lymph node and BAL are due to the kinetics of cell migration to and from these tissues.

As discussed in 7.1.4 T cells are key mediators of eosinophil infiltration, both directly through expression of IL-5 and indirectly through TNF-α-, IL-4- and IL-13-dependent stimulation of eotaxin secretion by tissue cells. It is therefore unsurprising that eosinophil infiltration is closely associated with lymphocyte infiltration. Further establishment of this association may have been possible by examining BAL IL-5 concentration by ELISA and/or the ability of BAL cells to express IL-5 by ELISpot.

### 7.3.5. Conclusion

Whilst the cytokine data in this chapter might provide useful mechanistic information, the degree of eosinophilia provides the answer to the question: "Does p7.IL-10 protect from disease induction?"[(Humbles *et al.*, 2004)]. Previous studies have successfully demonstrated protection from allergic asthma and associated eosinophilia[(Stampfli *et al.*, 1999)]. However, it is apparent that, in this study, coadministration of IL-10 with antigen does not protect from disease. The most likely explanation for this difference is that the former technique was able to generate a sufficient number of $T_R1$ cells, whilst the protocol used in this study was not.

The work in this chapter was originally intended to be relevant to the question of of allergic asthma. As described in the chapter introduction this, and other, models of asthma are of limited relevance to the human disease. In fact, this model may be more comparable to eosinophilic bronchitis than to asthma[(Brightling *et al.*, 2003)]. Another key caveat to such animal models is the use of a simple antigenic stimulant such as antigen-alum complexes. These may successfully trigger allergic inflammation but the complexity of real-world triggers of asthma and the importance of their innate immunostimulatory properties is becoming increasingly appreciated[(Akbari *et al.*, 2003; Kim *et al.*, 2008; Hammad *et al.*, 2009)].

The complexity of this disease is compounded by differences between various animal models, and between them and clinical asthma. Nevertheless, the work is relevant, as-is, to aspects of allergic airway inflammation.

# 8. General Discussion

## 8.1. The induction of regulatory T cells by IL-10

### 8.1.1. Inhibition of T cell activation by IL-10

The data in chapter 4 shows that proliferation and production of IL-2 and IL-4 in response to PMDD immunistion were dose-dependent and that the maximal response was obtained at a dose of 0.1 μg p7.OVA per mouse. This agrees with even the earliest studies, despite the use of different propulsion technology and bead size[(Eisenbraun *et al.*, 1993)]. It is important to note that, in these cases, an increased 'dose' corresponds to a higher copy number of plasmid on each gold particle. Therefore, rather than increasing the number of transfected cells, increased dose increases the copy number in each transfected cells. Maximal responses are likely the result of maximal expression which, itself, is likely the result of the saturation of the transcription/translation and/or antigen processing/presentation machinery of the cell.

The data in chapters 5 and 6 show that the coadministration of p7.IL10 reduces the immunogenicity of the OVA-encoding plasmid. As detailed in the introduction to this thesis, several mechanisms for this inhibition have been described: 1) Direct inhibition of T cells both by inhibition of CD28 signalling and by recruitment of the immunoinhibitory STAT3 transcription factor. 2) reduction of the number of antigen-presenting dendritic cells (DC) in the draining lymph nodes by inhibiting migration and inducing apoptosis. 3) Inhibition of antigen presentation by downregulation of MHC class II molecules, costimulatory molecules and cytokines via recruitment of STAT3. Which of these mechanisms is important under natural conditions, and which in this model, are important questions, the answers to which are likely to be at least partially dependent on the source of IL-10 in each context; dendritic cells, regulatory T cells or local tissue cells. In the case of PMDD immunisation both skin DC and local tissue cells such as keratinocytes may be transfected. Transfection of DC would, in theory, allow IL-10 to exert its effect on DC during and after maturation and migration as well as on T cells in the DC-T cell cluster. Transfection of keratinocytes may expose skin-resident DC to IL-10 until they migrate away from the site whilst lymph node T cells would not encounter the cytokine.

Figure 5-8 and Figure  show that the effect of coadministration of p7.IL10 is at least partially dependent on its expression in the same cell as p7.OVA. This somewhat agrees with earlier literature[(Morita *et al.*, 2001; Ross *et al.*, 2003)] that directly-transfected DC are key in PMDD immunisation (see 1.7.3). Autocrine, DC-derived IL-10 is known to maintain the cells in a hypoimmunogenic state[(Corinti *et al.*, 2001; Demangel *et al.*, 2002; Lang *et al.*, 2002b; Monteleone *et al.*, 2008)] and both naturally-occurring and artificially-transfected IL-10-secreting DC can also tolerise T cells to which they present antigen[(Takayama *et al.*, 1998; Takayama *et al.*, 1999; Akbari *et al.*, 2001; Chirdo *et al.*, 2005)]. In contrast, IL-10 produced by transfected keratinocytes would have only a very short time in which to affect skin DC as the danger signal provided by PMDD induces migration of both transfected and non-transfected DC away from the skin before maximal gene expression[(Porgador *et al.*, 1998; Bot *et al.*, 2000; Creusot, 2002)]. Therefore IL-10 expressed by directly-transfected DC

is likely the critical element in this model, allowing the cytokine to exert its inhibitory effects in all three ways described above.

### 8.1.2. Tolerance is not induced by IL-10

$T_R1$ cells were originally defined as IL-10-secreting regulatory cells which could be induced by their activation in the presence of IL-10[(Groux *et al.*, 1997)]. Indeed, the potential to induce $T_R1$ was central to the hypothesis that coadministration of IL-10-encoding vector could induce tolerance to antigen. The data in chapter 5 clearly show that IL-10 inhibited T cell activation by PMDD immunisation. Chapter 6 addressed the issue of tolerance induction. Initial experiments indicated that some degree of tolerisation did occur: The number of OVA-specific, cytokine-secreting T cells in the lymph node in response to a challenge with p7.OVA was lower in the group that were primed with p7.OVA and p7.IL10 than in the group that received p7.OVA alone. However, examination of the spleen showed that, at the time of challenge, the p7.OVA+p7.IL10 group had far fewer available OVA-specific DO11.10 T cells; as few as in control mice that received no antigen at all. Therefore it is possible, even likely, that the reduction in cytokine-secreting lymph node cells in response to p7.OVA challenge was simply due to a smaller pool of DO11.10 cells at the time of immunisation.

To examine this further fresh, CFSE-labeled DO11.10 cells were adoptively transferred prior to the p7.OVA challenge. Caution is required when comparing the results of this experiment to those involving only one cell transfer. Nevertheless the data clearly show no difference in the proliferation of freshly transferred cells between mice that were primed with p7.OVA alone and those that received coadministered p7.IL10. This important observation confirms that OVA-specific regulatory T cells were not induced by coadministered p7.IL10.

It could be suggested that the induction of $T_R1$ cells by IL-10 is an *in vitro*-only phenomenon[(Groux *et al.*, 1996; Fu *et al.*, 2008)]. Evidence for tolerance induced by injected recombinant IL-10 existed even before $T_R1$ cells were defined[(Rott *et al.*, 1994) (Enk *et al.*, 1994)]. However, it is unclear whether the mechanism behind these observations is the induction of regulatory T cells as opposed to the induction of anergy in antigen-specific T cells or skewing towards a less pathogenic $T_H2$ response. In fact, the majority of studies point to inhibitory effects of IL-10 on antigen presentation and antigen-presenting cells (see sections 1.3.2 – 1.3.4). Less and/or inferior antigen presentation to T cells would result in inexperience and/or anergy, not specifically to the induction of regulatory T cells.

It may seem contradictory that IL-10 may not be a key inducer of regulatory T cells when it is clear that $T_R1$ cells exist and that their regulatory properties are dependent on IL-10 (see 1.4.2). However it has not been demonstrated that $T_R1$ cells induce naïve T cells to become $T_R1$ cells. More likely, antigen-specific $T_R1$ cells inhibit antigen presentation and T cell activation at the DC: T cell cluster via the IL-10-dependent mechanisms described in the introduction to this thesis. Nevertheless, IL-10-secreting DC have been shown to protect against pathogenic immunity in several models (see 1.6.4), and there is evidence that these cells can induce the generation of $T_R1$ cells *in vivo*[(Akbari *et al.*, 2001; Wakkach *et al.*, 2003)].

The anatomy of the immune system might explain at least some of these discrepancies. Much of the work done on $T_R1$ cells was based on mucosal tissues and inflammation initiated by homeostatic expansion of adoptively transferred $CD4^+$ T cells. A theory put forward by Maynard and Weaver[(Maynard and Weaver, 2008)] and described in section 1.4.3 suggests that IL-10-secreting $T_{reg}$ play a specific role in regulating effector cells at the mucosal interface whilst $Foxp3^+$ $T_{reg}$ inhibit the activation of naïve T cells in the secondary lymphoid tissues.

It may also be the case that the induction of $T_R1$ cells is also anatomically restricted[(Duchmann *et al.*, 1995; Meiler *et al.*, 2008)]. There are several examples of protocols utilising IL-10-expressing DC which are tolerogenic using one administrative route but ineffective via another route[(Cua *et al.*, 2001; Oberholzer *et al.*, 2001; Zhang *et al.*, 2004; Xiao *et al.*, 2006; Pedersen *et al.*, 2007)]. Additionally, naturally-occurring tolerogenic DC have been described in mucosal tissues[(Akbari *et al.*, 2001; Chirdo *et al.*, 2005; Monteleone *et al.*, 2008; Ginhoux *et al.*, 2009)]. Tissue-specific non-immune cells such as skin keratinocytes[(Cao *et al.*, 2003; Dominguez-Castillo *et al.*, 2008)] and intestinal epithelial cells[(Iliev *et al.*, 2009)] appear to play a significant role in maintaining a tolerogenic environment at those sites. As stated in 1.5.3 it is possible that so-called 'specialised', tolerogenic DC are the result of their anatomical location.

The anatomical restriction of induction and function of IL-10-secreting $T_R1$ cells has implications for the model used in this thesis. Firstly, it brings into question the appropriateness of gene gun immunisation for IL-10-based induction of $T_R1$ cells: Whilst there is evidence that IL-10-transfected DC can induce $T_R1$ cells, this function appears to be largely restricted to mucosal tissues. Nevertheless, tolerisation via the skin has been shown in the literature and it is possible that IL-10-expressing skin DC may induce the generation of $T_R1$ cells. Secondly, examination of T cell activation in the lymph node may not be relevant to the examination of $T_R1$ induction by IL-10: It is not clear that the induction of $T_R1$ cells occurs in the lymph nodes and both effector and regulatory T cells share similar proliferative characteristics during their response to antigen[(Adler *et al.*, 2000)].

### 8.1.3. Asthma

As discussed above, examination of T cell activation in the lymph node may not be the ideal readout for the tolerisation of DO11.10 cells in this model. Whether the induction of OVA-dependent experimental asthma can be inhibited is perhaps more appropriate. Noninflammatory administration of antigen has shown some limited tolerogenic success in similar models[(Barbey *et al.*, 2004; Vissers *et al.*, 2004; Van Hove *et al.*, 2007)]. The adminstration of IL-10-treated and IL-10-expressing dendritic cells (introduced in 1.6.3 and 1.6.4, respectively) have been demonstrated to inhibit inflammation in models of OVA-dependent airway inflammation[(Koya *et al.*, 2007; Henry *et al.*, 2008)]. Nevertheless, the data in chapter 7 shows that PMDD coadministration of OVA and IL-10 does not protect against disease.

It is understood that allergic asthma is primarily a $T_H2$ disorder and that $T_H1$-biasing genetic vaccines have been demonstrated to be protective[(Lee *et al.*, 1997; Maecker *et al.*, 1997; Maecker *et al.*, 2001; Ludwig-Portugall *et al.*, 2004)]. The aim of this study, however, is to examine T cell tolerisation; a different solution to this disease. Because gene gun immunisation is more $T_H2$-inducing than plasmid injection it can exacerbate airway inflammation[(Bartholdy *et al.*, 2004; Scheiblhofer *et al.*, 2007)]. Therefore any suppressive effects of a gene gun-based

vaccine would almost certainly be due to the gene(s) being transferred and not to the immunomodulatory effects of the protocol itself.

Lymphocytes, as opposed to mast cells, are key in the induction of asthma[Brusselle *et al.*, 1994]. Therefore the induction of regulatory T cells that control antigen-specific responses is certainly an appropriate approach to abrogating disease. Indeed, inadequate regulatory T cell function likely plays an important role in the development of asthma [Akdis *et al.*, 2004; Jaffar *et al.*, 2004] whilst both $CD4^+CD25^+$ regulatory T cells and IL-10-secreting $T_R1$ cells [Akbari *et al.*, 2002; Akdis *et al.*, 2004; Kearley *et al.*, 2005; Lewkowich *et al.*, 2005; Kearley *et al.*, 2008] have been shown to inhibit disease. Therefore, in agreement with earlier chapters, the failure of the treatment used in this project to protect against asthma suggests that it does not induce the generation of regulatory T cells.

## 8.2. Implications for PMDD-based immunotherapy

### 8.2.1. Limitations of this study

A significant gap in this study is the range of cytokines examined in harvested cells. In particular, it would have been interesting to examine the effect of coadministration of p7.IL10 on IL-5 and IL-13 expression by DO11.10 cells, especially in the longer-term experiments. In general, advances in immunoassays such as multiplex sandwich ELISA and microbead-based assays now allow wider screening of cytokine expression. Utilisation of such an assay may have provided a clearer understanding of the response.

Although phenotyping of regulatory T cells is still far from perfect, flow cytometric analysis of Foxp3 expression has proven very useful and is now routine. Such analysis would have been an insightful addition to the flow cytometry performed in this study.

Finally, repetition of some experiments using IL-$10R^{-/-}$ DO11.10 donor mice, and perhaps also in IL-$10R^{-/-}$ balb/c recipients, might increase confidence that the suppressive effects observed were directly attributable to coexpressed IL-10.

### 8.2.2. Future studies with IL-10

Firstly, there is evidence that substitution of mammalian IL-10 with viral IL-10 might improve the immunosuppressive effect of the coadministered p7.IL-10 plasmid[Takayama *et al.*, 2001b; Moore *et al.*, 2004].

The question of direct transfection of, vs cross-presentation by, DC has been discussed above and in earlier chapters. Section 1.7.3 describes the three mechanisms likely to be involved in naked DNA immunisation. Clearer answers might be provided by the restriction of antigen expression to dendritic cells by placement of the *ova* gene under the control of a DC-specific promoter such as that of fascin or dectin-2. Similar information might be obtained by the use of $OVA_{cyt}$, a mutant ovalbumin protein whose secretion signal has been removed and which remains largely confined to the cytoplasm, rather than being secreted into the extracellular milleu.

Whether the effect of IL-10 is directly on T cells or via an effect on DC might be examined further by the use of IL-$10^{-/-}$ DO11.10 T cells. Similarly, the repetition of the original *in vitro* work by H. Groux *et al*., but substituting a non-APC stimulator such as anti-CD3 antibodies, would illustrate the importance (or lack thereof) of the effect of IL-10 on APC in the IL-10-dependent generation of $T_R1$ cells[(Groux *et al.*, 1997)].

One of the explanations proposed earlier for p7.IL10 not inducing tolerance is the kinetics of expression of each plasmid relative to the other. Placement of *il10* under the control of a promoter with a more extended expression profile may enable the cytokine to persist until presentation of OVA has ceased. This can also be extended to the antigen gene, *ova*. Other studies have shown that placing the antigen gene under control of an APC-specific promoter greatly reduces the efficiency of both injected plasmid and gene gun immunisations[(Corr *et al.*, 1999; Cho *et al.*, 2001; Hon *et al.*, 2005)]. When developing a tolerisation protocol, however, this may be desirable. Section 1.7.3 describes the three mechanisms likely to be involved in naked DNA immunisation. Restriction of antigen expression to dendritic cells would ensure that only directly-transfected DC would present antigen and, in the case of linked p7.IL10 coadministration, that all OVA-presenting cells coexpress IL-10. This effect could be further guaranteed by the use of $OVA_{cyt}$, a mutant ovalbumin protein whose secretion signal has been removed and which remains largely confined to the cytoplasm, rather than being secreted into the extracellular milleu.

### 8.2.3. Other molecules / techniques

Since IL-10 exerts many effects via its action on DC, the pathway downstream of IL-10 is an appropriate target for immunosuppressive treatments. Gene gun immunisation is mediated mainly by directly-transfected DC and is therefore a good tool for exploration of intracellular immunomodulatory molecules. Therefore coadministration of the gene encoding STAT3, a transcription factor activated by IL-10, may bypass the need for cytokine expression and detection and directly activate the DC antiinflammatory response described in 1.3.4. Even further downstream, transfection with SOCS3 might induce tolerogenic effects on DC[(Li *et al.*, 2006)].

The maturation status of DC has been suggested to play a role in activation vs tolerisation of T cells (see 1.5.1). Coadministration of molecules that interfere with the maturation of DC, such as iκB and CTLA-4 may result in tolerogenic presentation of antigen[(Tan *et al.*, 2005)].

As described in the introduction to this thesis the gene gun, and genetic immunisation in general, provides a platform through which encoded proteins can be administered with relative ease. Therefore more recently discovered immunomodulatory molecules such as IL-35 and galnectin-1, about which relatively little is known, could be examined as candidates for inclusion in a tolerogenic vaccine[(Collison *et al.*, 2007; Collison *et al.*, 2009; Ilarregui *et al.*, 2009)].

Despite the plethora of available molecules, one further hurdle remains. This study has examined prophylaxis; vaccination to prevent future immune responses. In the context of pathological immunity, the ability to interfere with an ongoing response is more desirable. Unfortunately, experienced T cells

are more resistant to tolerisation than are naïve T cells and ongoing responses can make tolerising protocols ineffective or even detrimental(London *et al.*, 2000; Hurst *et al.*, 2001).

Although an effective inhibitor of immune responses, it is unlikely that coadministration of IL-10 in a gene gun vaccine is an appropriate method for the induction of tolerance and/or regulatory T cells, especially in remote tissues such as the lung. The gene gun remains a very flexible immunisation tool, allowing the continued study of immunomodulatory molecules in the search for an effective immunosuppressive vaccine.

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